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Studies on the etiology of stunting syndrome in turkey poults

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Studies on the etiology of stunting syndrome in turkey poults

by

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For the Graduate college

DEDICATION

This dissertation is dedicated to my parents and teachers. Without their unconditional love, guidance, and succor in every part of my life, I would not have made it to this point.

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CHAPTER 1. GENERAL INTRODUCTION

Infectious diseases of the gastrointestinal tract (GI) are not uncommon in poultry. The general outcome of these diseases is poor performance leading to economic losses. An enteric disease syndrome (complex) of young poultry has been reported under a variety of names both in chickens ["malabsorption syndrome" (216, 223), "infectious stunting syndrome" (18), "broiler runting syndrome" (187), "pale bird syndrome" (72) and "helicopter disease" (208)] and turkeys ["turkey viral enteritis" (208), "poult enteritis" (208), "malabsorption syndrome" (192), "stunting syndrome" (3, 18), and "runting and stunting syndrome" (149)]. In most of these cases, the name(s) reflects the outcome and/or clinical characteristics of the disease rather than the specific etiology. One of the characteristics common to these disease syndromes is the presence of enteritis (209). This common characteristic led to a common name, the "enteric disease complex". Although, there are a number of enteric conditions in poultry which are characterized by the involvement of a specific etiologic agent (including viruses, bacteria, parasites, etc.), many of the disease syndromes included in the "enteric disease complex" have undetermined etiology (209). Since the etiologic agent(s) is unknown, it is difficult to ascertain whether these enteric disease syndromes are one and the same, related somehow or are entirely different entities. Stunting syndrome (SS) is an enteric disease syndrome which is included in the "enteric disease complex". Stunting syndrome has been reported in both chickens and turkeys (208, 209). The syndrome produces similar clinical manifestations in both species, however, the specific etiologic agent(s) has yet to be determined. Until the etiologic agent(s) is identified, it will continue to be very difficult to differentiate and/or discriminate this disease condition in either species.

Stunting syndrome in turkey poults occurs during the first four weeks of their life (18, 192, 208). There is poor feed utilization, decreased weight gain,

maldigestion (decreased intestinal disaccharidases activity), malabsorption and poor feather development. At necropsy, the intestines are distended with gas and fluid with pale discoloration. The intestines appear flaccid. The ceca are distended with yellow-to-brown frothy contents (18, 149, 192). The affected flocks usually show high morbidity (stunting) but the mortality is variable. The stunted poults give the flock an uneven appearance. Poults remain smaller (runted) throughout the grow out periods.

The reported experimental observations strongly suggests that SS is an infectious disease. No specific etiology has been determined yet. There are numerous viruses that cause specific enteric disease (e.g., enteritis due to coronaviruses, rotaviruses, astroviruses, caliciviruses, enteroviruses, enterovirus-like viruses, parvoviruses, parvovirus-like viruses, reoviruses, etc.) in poultry (208, 209). Similarly, bacteria, parasites and other factor(s) can cause enteric disease. It has been speculated that these agents play a role in the "enteric disease complex" and, in some cases, they have been incriminated as being the underlying cause. However, these agents have not been associated specifically with SS.

The "enteric disease complex" poses a unique problem. Since the syndrome occurs in different avian species and the majority of the reported observations stem from clinical cases rather than from experimental studies, it is difficult to have one representative experimental model. During the past few years, considerable progress has been made towards defining a model for SS. According to this model, day-old turkey poults inoculated with a SS inoculum developed stunting and diarrhea (3). The disease was assessed by various physiological parameters including decreased intestinal disaccharidases (enzymes involved in disaccharides digestion) activity. Electron microscopic (EM) examination of intestines from SS affected poults revealed long segmented filamentous organisms (LSFOs) attached to the intestinal epithelial cells (4). Subsequent filtration studies revealed that

bacteria were not the primary cause but did accentuate the outcome of the disease. It was speculated that a virus(es) was the primary etiologic agent(s).

In order to understand the pathogenesis of the disease, develop diagnostic, prevention and control methods, it is of paramount importance to identify the etiologic agent. A proper association of disease with a definite etiology helps in defining the disease, host species involved, prevalence of the disease, association/predisposition with other diseases, etc. Unless the etiology of a disease is known, defining protocols/procedures for the control and prevention can be very difficult. The objectives of the present study were to isolate and identify the etiologic agent(s) involved in SS. The specific aims included:

1. Isolation and identification of the etiologic agent.
2. Development of a system(s) for the isolation and *in vitro* propagation of the etiologic agent.
3. Determining the relatedness of the agent with known agents.
4. Determining the physico-chemical properties of the agent.

Dissertation organization.

The main body of this dissertation is comprised of five chapters. Each chapter represents a complete manuscript. The manuscripts are written in concordance with *Avian Diseases* format. Each manuscript has its own abstract, introduction, materials and methods, results, discussion and acknowledgment section. The manuscripts are preceded by a general introduction and a literature review. A general discussion and references (cited in general introduction, literature review and general discussion) are included at the end of the dissertation. Akbar Ali is the primary author and principal investigator of these studies.

CHAPTER 2. LITERATURE REVIEW

This literature review comprises a general overview of "enteric disease complex/poult enteritis/stunting syndrome" in chickens and turkeys. The reports on SS in chickens were more numerous in the late 1970s and early 1980s. There are considerably more reports on this disease in chickens than in turkeys. However, within the past few years, the reports regarding SS in chickens have subsided significantly. The defining/discriminating characteristics between chicken and turkey SS has not been defined yet and the problem continues to exist in turkeys. Although there are recent reports about SS in turkeys, the information is limited. Therefore, SS of both chickens and turkeys is reviewed. A brief review of those infectious agents, which have been reportedly alleged to occur in association with this syndrome, is also presented. This alleged association is largely based on their demonstration by electron microscopy (EM) in the feces of birds with enteritis and diarrhea.

The concept of an avian disease caused by a mammalian pathogen and vice versa is not new. Examples include salmonellosis (137), influenza (9, 228, 229), etc. The potential for other species to act as a reservoir for infectious agent(s) that potentially causes SS in turkeys should be considered. Those viruses that morphologically look like SSA, have also been reviewed.

Stunting syndrome in chickens.

The history of SS dates as far back as 1947 when "stunted chick" disease (similar to what is now known as SS) was reported for the first time (212). The exact description of the disease was reported in 1977 in US (181), in 1977 in Australia (200), and 1978 in Netherlands (126). The disease was described as runting and leg weakness in broilers. Since then, the disease has been described around the world under different names such as pale bird syndrome (72, 135, 176), infectious

stunting syndrome (18), runting syndrome (127, 187), stunting syndrome (223) and malabsorption syndrome (216, 223).

Age susceptibility. The susceptibility of chickens to SS infection is highly age related. Day-old chickens are highly susceptible, while the susceptibility declines rapidly after 3 days of hatch and birds are no more susceptible after 14 days of age (111, 140). In one experiment broiler chicks, inoculated at 1 day of age with SS inoculum, developed all the clinical signs (see below) of the disease. Broiler chicks inoculated at 3 and 7 days of age did develop the disease but stunting was less pronounced. However, chicks inoculated at 14 days of age did not develop the clinical disease. (127, 269). These experimental findings corroborate field observations (18, 199).

Experimental disease transmission. The SS in chickens can be reproduced experimentally by orally inoculating them with the crude intestinal homogenate prepared from the intestines of affected chickens (16, 126). The criteria for successful experimental transmission in chickens varies widely. The most commonly used parameters are growth rate depression, clinical signs, lesions, maldigestion and malabsorption (140). The experimental transmission of SS through contact exposure has also been reported in chickens (269, 284) with 100% infection rate.

Etiology. Bacteria-free filtrates of the intestinal homogenate are capable of transmitting some aspects of the disease in chickens (126, 140) but not the total syndrome. Inoculation of chickens with a bacteria-free filtrate resulted in elevated serum alkaline phosphatase levels and reduced growth rate but the osteoporitic changes could not be reproduced (126). It has also been reported that the infectivity lies in the sediment following ultracentrifugation of the intestinal homogenate. Attempts to reproduce the disease with aerobic and anaerobic bacteria isolated from this sediment were unsuccessful. However, the disease could be reproduced

by mixing the bacteria with bacteria-free-filtrate (140).

Earlier endeavors to reproduce the disease experimentally using either isolated (from the inoculum) bacteria, viruses and/or both resulted in no, or very little success (127, 129, 165). The viruses which have been observed from SS affected chickens were reoviruses (102, 129, 146, 269), coronavirus-like virus(126), enterovirus-like viruses (67, 159, 165), parvoviruses (113), rotaviruses and adenoviruses (44, 146). The Koch's postulates were not fulfilled with any of the aforementioned viruses. Among the bacteria, long segmented filamentous organisms (LSFOs) and *Campylobacter sp.* (140) have been observed in SS affected chickens but their exact role in the disease has not been determined.

Clinical disease. The clinical manifestations of this disease vary but the common expositions include poor growth (runting/stunting), poor feed utilization, depression, spradled wing feathers, diarrhea and enteritis (140, 208). Pasted vents and diarrhea have been observed during acute stages of the disease (18). The affected chicks appear unhealthy with hunched back appearance, ruffled feathers, drooping wings and preferentially eat litter rather than feed (59). The shanks appear pale (hence "pale bird syndrome"). Mortality is variable, but morbidity is high. The recovered flocks appear active but a percentage of chicks are very small, thus, giving the flock an uneven appearance.

Necropsy lesions. In chickens, the necropsy lesions include enlargement of the proventriculus, loss of pigmentation, pancreatic changes and catarrhal enteritis. (128, 184, 199). The intestines are distended with poorly digested feed and watery contents (16, 184, 217). The intestines appear pale with varying degrees of enteritis (16, 18, 184, 217). The ceca are frequently filled with yellow/orange mucoid feces and gas (187). The pancreatic changes in the affected chickens include atrophy, and fibrosis of one or more of lobes (28, 59, 140, 179, 184, 198, 199). The reported skeletal abnormalities, although not consistent with all the reports, include rickets

and osteoporosis. Femoral head necrosis may also be associated with the syndrome but is not consistent (100). The atrophy of lymphoid tissues, bursa of fabricius and thymus, is a common occurrence in affected chickens (16, 179, 184, 187). Encephalomalacia has also been reported in association with SS in broiler chickens (187).

Histopathology. Histopathological and ultrastructural changes of the intestinal tract from chickens affected with SS include degeneration and necrosis of enterocytes in the crypts of Leiberkühn, infiltration of macrophages and lymphocytes in the lamina propria and cystic degeneration (cuboidal epithelial cells surrounded by a basement membrane) (18). The microscopic lesions of the pancreas include loss of acinar cells, proliferation of intralobular ductules and obstruction to pancreatic drainage (59, 140, 241). Histopathological lesions of bones in affected chicks include enlargement of the epiphyseal zones of proliferation and tibial dyschondroplasia (126, 127, 269). The atrophied bursa and thymus shows marked depletion of cortical lymphocytes when examined microscopically (16, 179, 184, 187). Proventriculitis and/or proventricular distention in association with SS has been attributed to dilated proventricular glands and interstitial fluid accumulation (126, 127, 184).

Immune system. The immune competency of birds affected with SS has not been extensively studied. There is one citation about reduced cell mediated immune response by the SS affected chickens (260). However, humoral immunity remained unaltered. The suppression in cell mediated immunity was very transient during the infection and was also highly weight related. But it has not been resolved whether the alterations in the immune response are due to the effects of SS or malnutrition (disease outcome) on the primary reticuloendothelial system. Because, the thymic lesions observed during SS are histologically indistinguishable from those caused by nutritional stress (90).

Stunting syndrome in turkeys.

SS in turkeys continues to be a problem. Analogous to SS in chickens, SS in turkeys has been reported under different names. Turkey flocks affected with SS display alterations similar to what has been observed in SS affected chickens except there tends to be higher mortality and morbidity in turkey flocks. The disease also varies in the degree of severity. SS is less severe in poults than broilers (18). Turkey poults within the first 6 weeks of life, and more specifically 1 to 3 weeks of age are highly susceptible. SS infected turkey poults gain less weight and have poor feed conversion ratios. The affected poults are depressed and experience diarrhea (149, 192, 208, 261). There are alterations in the development of primary feathers of the wings giving poults a "helicopter" appearance. The frequency of rickets is lower in poults than in chickens, however, it is more pronounced when it occurs. There is hypocalcemia (attributed to malabsorption) which progresses to a rachitic lesion associated with vitamin D depletion and hypophosphatemia. There is increased incidence of angular limb deformities in recovered poults. The affected poults remain small, as compared to unaffected poults, throughout the growth period. The necropsy lesions include gaseous and watery intestinal contents, dilated ceca with frothy yellow-to-brown contents, and pale and atonic (thin) intestinal tract (18, 149, 192, 208, 261). There is maldigestion due to decreased intestinal disaccharidases activity (3).There is mild infiltration of inflammatory cells in the intestine. Villus atrophy and cryptic hypertrophy has also been observed histologically.

SS in turkeys can be reproduced experimentally by orally inoculating them with the crude intestinal homogenate prepared from the intestines of affected turkeys (3, 261). The criteria used for successful experimental transmission includes growth rate depression, clinical signs, lesions, maldigestion and malabsorption (3).

The etiology of SS in turkeys has not been previously reported. Long

segmented filamentous organisms have been found attached to intestinal epithelial cells of SS affected poults (4). However, bacteria-free filtrates of the intestinal homogenate were capable of transmitting SS in turkeys (231) but the severity and longevity of the disease was diminished. The underlying cause was speculated to be viral.

Etiology of enteric infections.

A specific etiologic agent for the "enteric disease complex" remains undefined, but a number of infectious agents have been incriminated with this syndrome. Among these, viruses are considered to be the most probable etiology. In chickens the association of virus(es) with SS has been on the basis of direct electron microscopic examination of feces, ultrastructural identification in tissue sections and, in a few cases, virus isolation in cell culture and/or embryonated eggs. In many of these cases the cause-and-effect relationship has not been established. There is some evidence that bacteria as well as parasites such as cryptosporidium may be involved. The contribution in the causation, development and progression of SS by the non-infectious agents such as toxins, nutrient(s) deficiencies etc., has not been ruled out. Of all these, no satisfactory etiologic agent(s) has been established.

Viruses.

Coronaviruses. The members of coronaviridae family affects both mammalian and avian species. Among the coronaviruses that infect poultry, infectious bronchitis virus (IBV) of chickens (123) and bluecomb disease virus (often called turkey coronavirus; TCV) of turkeys (194) are of major economic concern. Although the members of coronavirus occur in the intestinal tract of both chickens and turkeys, coronaviral induced enteritis has been thought to be a problem of turkeys only. Bluecomb disease is a highly infectious disease of turkeys of all age groups causing very high morbidity (diarrhea, depression, reduced weight gain and enteritis) (194). This disease has also been referred to as mud fever, coronaviral

enteritis, transmissible enteritis and infectious enteritis (194). The coronaviruses have also been observed in feces from diarrheic turkeys (Quebec isolates; 38, 39).

The coronavirus responsible for bluecomb disease (Minnesota reference strain) appear as pleomorphic (50-150 nm) viruses surrounded by a membrane studded with petal shaped projections (211). The virus has a buoyant density of 1.16-1.24 g/ml in sucrose (185). Chloroform treatment at 4 C for 30 minutes results in complete inactivation of the virus but the virus is resistant to a pH of 3.0 at room temperature for 30 minutes (47). The virus is also resistant to a temperature of 50 C for 1 hour even in the presence of 1M magnesium chloride (47). Both Quebec and Minnesota isolates of turkey coronavirus have been shown to agglutinate erythrocytes from rabbit and guinea pig but not from cattle, horse, sheep, mouse, goose, monkey and chickens (38).

The TCV has been successively propagated in embryonated turkey (>15 days of age) and chicken (>16 days of age) eggs when inoculated via amniotic cavity (1, 174, 185, 188). Propagation in embryos does not alter its pathogenicity for turkeys even after several passages. Attempts have also been made to propagate the bluecomb agent in intestinal organ culture. The virus survives in this system for a period of 120 h without any apparent multiplication (2). There is one report of successful propagation of TCV (Quebec isolates and Minnesota strain) in an established cell line (HRT-18) derived from human rectal carcinoma cells by adding trypsin in the maintenance medium (40, 42). Virus replication caused syncytia formation. A positive identification was made on the basis of immunofluorescence, EM and hemagglutination properties of the virus. Other cell culture systems investigated were from primary kidney (of chickens, turkeys, quail and monkey) and liver (chickens and turkeys) cells but without any success (47).

The TCV is antigenically different from other mammalian (transmissible gastroenteritis virus, bovine coronavirus and mouse hepatitis virus) and avian (IBV)

coronaviruses on the basis of hemagglutination inhibition (HI) assay (194). However, different TCV isolates are antigenically related among each other. It has also been demonstrated by immune EM, HI and western immunoblotting techniques, that different cell culture adopted (Quebec) isolates were closely related to bluecomb agent (Minnesota reference strain) and also to bovine coronavirus (Nebraska calf diarrhea virus) (41). These observations await further confirmations. The same authors have also reported a close similarity between TCV (Quebec and Minnesota strains) and bovine coronavirus (Nebraska calf diarrhea virus) but no relation to IBV or TGEV on the basis of cDNA hybridization experiments (41).

The TCV, responsible for bluecomb disease, affects turkeys of all age groups but chickens and pheasants are refractory to the infection (103). The necropsy lesions are primarily confined to the intestinal tract. The intestines are distended with frothy yellow-to-brown contents (194). The intestinal lesions are short lived in infected poults. The lesions normally appear at 24 hours post inoculation (PI), peak at 3 days PI and start regressing by 5 days PI. By 10 days PI, the intestinal tract appears normal (195). Similarly, the viral antigen has been detected in the intestinal epithelium (using immunofluorescence test) as early as 12 hours PI and persisted upto 336 hours PI (196). The villi return to their normal appearance by 120 hours PI. The severity of disease varies with respect to the presence of other organisms in the intestinal tract. Poults inoculated with a mixture of intestinal filtrate (from affected poults) and common enteric bacteria suffer heavier economic losses than those inoculated with filtrate alone suggesting that the intestinal microflora alters the severity of disease (175).

The role of TCV in poult enteritis other than the bluecomb disease has not been fully explored. A survey of diarrheic turkey flocks conducted (on the basis of viral morphology under EM) in the US failed to detect any coronaviruses (204, 205, 219). However, the viral pleomorphism may account for the negative results. On the

other hand, coronaviruses antigenically similar to bluecomb agent (Minnesota reference strain) were detected in diarrheic turkey flocks in Canada. A 47.5% of 114 flocks tested were positive for coronaviruses on the basis of ELISA and immune EM (39).

The role of coronaviruses as the causation of SS has not been reported. The difficulty of identifying coronaviruses by EM due to their pleomorphic morphology, lack of in vitro system(s) to isolate and propagate the enteric coronaviruses, etc., are some of the major problems in defining the role(s) of coronaviruses in poult enteric disease complex and/or SS.

Astroviruses. Astroviruses, members of the family *Astroviridae*, have been isolated from both humans and animal species. In most of the species, the astroviruses are primarily associated with gastroenteritis. Among avian species, astroviruses have been detected only in turkeys. The term astrovirus was coined in 1975 to describe its star-shaped morphology when examined by direct EM (Latin word "astron" meaning star-shaped) (139). It appears as a small round virus with distinctive 5 or 6 pointed-star-like appearance. Typically, less than 10% of the astrovirus display the star-shaped morphology (156). They are approximately 30 nm in diameter.

Attempts for *in vitro* propagation of astroviruses have met with variable success. Most of the astroviruses of animal origin have not been isolated *in vitro* with the exception of some swine and bovine isolates. The human isolates have been successfully propagated in HEK (human embryonic kidney), LLC-MK_2 (rhesus monkey kidney epithelial) and Caco-2 (human colon carcinoma) cells in the presence of trypsin (134). Some of the isolates of swine and bovine astroviruses have been propagated in embryonic swine kidney (237) and primary neonatal bovine kidney cells (8) respectively. One of the characteristic feature for their *in vitro*

propagation is their requirement for trypsin in the medium. Attempts to grow the turkey astroviruses *in vitro* have been unsuccessful.

The physicochemical properties of the avian astroviruses have not been reported. However, the human astroviruses are stable at pH 3.0 (132). They are resistant to chloroform, a variety of detergents and lipid solvents (130) and retain their infectivity after 5 minutes at 60 C. The human astrovirus particles appear to be stable when stored at -70 C for 6-10 years (275). The virus has a positive sense, single stranded RNA genome. They have a density of 1.35-1.37 g/ml in cesium chloride (143).

Astroviruses have been identified in a wide variety of young mammals, humans and poultry including lambs (242), calves (278), deer (265), piglets (22, 237), mice (124), dogs (141), ducks (82), and turkey poults (156, 204, 207). Astroviruses appear to cause infection in a species specific manner (132). So far 7 serotypes have been identified in humans (131, 143) and 2 in bovine (281). In poultry, astrovirus infections have been limited to turkeys and have not been detected in chickens. In turkeys, the astroviruses were first identified in 11-day-old turkey poults with diarrhea and high mortality (156). Subsequently, astroviruses have been observed in flocks of young turkeys in the US in 1985 (219) and 1986 (203). Typically, the infection occurs between 1-3 weeks of age. Clinical signs during natural outbreaks in turkey poults include diarrhea, enteritis, listlessness, litter eating and nervousness (208). In affected poults the mortality is usually low but the morbidity (stunting) is very high. The enteric disease has been reproduced by the turkey astroviruses in susceptible poults under experimental conditions (205, 256, 257). The infected poults gain less weight. There is maldigestion (impaired intestinal disaccharidases activity) (257) and malabsorption (reduced D-xylose absorption) (208). The insult to the intestinal tract, following viral infection, was obvious within 3 days PI and peaked between 5 to 7 days PI. By 14 days PI, the

clinical disease was no longer evident and the intestines appeared normal. At the peak infection, the intestines were thin containing gaseous fluid. The ceca were dilated with yellow-to-brown frothy contents. Histologically, astrovirus infection induced intestinal crypt hyperplasia which was first evident in jejunum and, as the disease progressed, the changes appeared in duodenum and ileum also. Ultrastructural examination of the villous enterocytes revealed intracytoplasmic crystalline arrays of astroviruses (256).

Under field conditions, the astroviruses are rarely identified as the sole viral agent but most commonly are associated with group D rotaviruses (204, 205, 219). When there is a mixed infection, the astroviruses are the first one to be detected during the course of infection (208). It has been reported that the rotaviruses were not detected until 7 days PI, whereas astroviruses were detected at 4 days PI. The astroviruses, thus, are considered as mild pathogens when alone but their role, however, may become more important in situations where another enteropathogen is involved.

Rotaviruses. Rotaviruses are a leading cause of viral diarrhea in humans (118) and other mammalian neonates (57, 221). Rotaviruses are the single most important etiologic agents of viral diarrheal illnesses of infants and young children with an average death rate of about 3.3 million per year world wide (14). Rotaviruses of avian origin were first reported in 1977 from a flock of turkey poults experiencing diarrhea (208). Since then, there have been numerous reports of rotaviruses being identified from the feces of a number of avian species including chickens (152, 153, 155, 157, 286), turkeys (154, 155), pheasants (83, 84, 206), ducks (251), pigeons (167) etc. Rotavirus infections in avian species (either alone or in combination with other infectious agents) are frequently associated with enteritis and diarrhea.

Rotaviruses comprise a genus in the family *Reoviridae* and have a distinctive morphological appearance. Complete particles measure approximately 70 nm in diameter and have a characteristic double layer protein capsid (icosahedral) arranged in such a way as to give the virus a smooth outer surface while retaining its spoke-like internal appearance thus the name rota- (Latin word rota mean "wheel"). Within the inner capsid lies a third protein layer called the core which encompasses the viral genome consisting of 11 segments of double stranded RNA (58). On the basis of morphological and physical characteristics, rotaviruses have two forms; single-shelled and double-shelled. The particles having both (inner & outer) the capsid layers present are generally termed as double shelled (smooth or complete) particles and are about 65-75 nm in diameters. When the outer capsid layer is absent, the particles appear rough and are referred to as single shelled, incomplete or rough particles. The incomplete viral particles measure about 10 nm less in diameter than the complete viral particles. Double shelled particles can be converted to single shell and core particles using chelating and choatropic agents (58). These different rotavirus particle types possess different biophysical and biological properties. For example, the infectivity depends upon the presence of the outer capsid layer and its removal results in loss of infectivity (21, 51, 54, 56). When separated on cesium chloride gradients, the complete, incomplete, and core particles have a density of 1.36, 1.38, and 1.44 gm/ml respectively (15, 21, 54, 214).

The physico-chemical properties of avian rotaviruses have not been extensively studied. However limited available information, on two turkey isolates, reveal its stability to treatments with pH (3.0 pH for 2 hours) and chloroform. There is 100-fold drop in the infectivity when subjected to heat treatment at 56 C for 30 min. (116). A pigeon rotavirus isolate was stable to ether, chloroform and deoxycholate treatment (167). The buoyant density of single and double shell particles in cesium chloride is 1.34 and 1.36 g/cm^3 respectively (116). A pheasant

group D isolate had a buoyant density of 1.347 and 1.365 for double-shelled and single-shelled particles (48). The average size for this pheasant group D rotavirus was determined to be 80 nm for double-shelled particles and 70 nm for single shelled particles. Avian group A and group D rotaviruses hemagglutinate erythrocytes from different species (48, 95, 116, 167)

Rotaviruses have been classified into groups, subgroups and serotypes. The classification into groups has been based on their antigenic relationship and migration patterns of their segmented genome when subjected to electrophoresis (189, 190). A rotavirus serogroup includes viruses that share cross-reacting antigens detected by a number of serologic tests such as ELISA, immunofluorescence, and immune electron microscopy (189). The group determinants (common antigens) are predominantly viral structural proteins and may contain some non-structural proteins as well. The viral protein-6 (VP 6), for example, is the predominant group antigen as it constitutes 51% of the virion mass (144). VP 6 also mediates subgroup specificity (117, 144). Classification into serotypes is based on the reactivity of viruses in plaque reduction or neutralization assays (105, 106, 274) and measures antibody reactivity to VP 4 and VP 7. These two proteins constitute the outer capsid of the virion. Serotypes are further divided into G-serotypes and P-serotypes on the basis of the nature of VP 4 and VP 7 (87, 105, 106, 233). At present, the rotaviruses have been divided into 7 antigenic groups which are designated as A, B, C, D, E, F and G. Group A, B, and C rotaviruses have been found in both humans and animals, whereas, the others have only been found in animals. Rotaviruses of avian origin have been placed in groups A, D, F and G (160). In earlier literature, non-group A rotaviruses of turkeys were reported as rotavirus-like but later on they were found to be the members of group D rotavirus (163, 208, 221, 254, 255). Rotavirus of group A and D have been found in chickens, turkeys, and pheasants (163, 208). Group F rotaviruses have been

detected from chickens and turkeys and group G from chickens only (221). Group D rotaviruses are more prevalent in avian species whereas group A rotaviruses are predominant in mammals. The group D rotaviruses were reportedly found in 58% of turkey flocks (10-to-21-day-old diarrheic poults) surveyed (219). In another study, 67% of the flocks with enteric disease (diarrhea, enteritis, "poult enteritis") were positive for Group D rotaviruses when compared to normal flocks (26%). However, the prevalence of group A rotaviruses was 26% and 22% in healthy and diseased flocks respectively (204). One of the most common observations made during these surveys was that group D rotaviruses were rarely identified as the sole viral agents. Most commonly, the flocks were co-infected with astroviruses (204, 208, 215, 219). The presence of group A and group D rotaviruses in broiler breeders was similar, as the sero-prevalence rate was 63 and 70% respectively (160). The role(s) of the rotaviruses in the stunting syndrome has not been reported.

The group D rotaviruses most commonly affect poults during the first four weeks of life. Rotaviruses have been detected in poults as young as 3 days of age. In an experimental situation where the inoculum was a combination of astroviruses and group D rotaviruses, the rotaviruses were not detected until 7 DPI, whereas, the astroviruses were detected after 4 DPI (205). In general, rotavirus infections in poults cause diarrhea with dilated intestines, watery gut contents and impaired intestinal absorptive capabilities. Histological lesions typical of rotaviral infections have been observed. These include basal vacuolation of enterocytes, separation of enterocytes from the lamina propria with subsequent desquamation, villous atrophy, enlargement of lamina propria, scalloping of the villous surface, fusion of villi and leukocytic infiltration of the lamina propria. In general, mean villus lengths were decreased and crypt depths increased following experimental infection, resulting in significantly decreased villus to crypt ratio (289). Chickens inoculated with group A rotavirus develop either very mild, or no clinical signs of the disease (158). Laying

chickens infected with group A rotaviruses had a transient decline in egg production (158, 287, 288, 289, 290). The role of group D rotaviruses as the sole agent for causation of infection in poults has not been reported. They cause more severe disease in combination with astroviruses than astroviruses alone. Experimental infection of chickens with group D rotaviruses produced mild clinical signs. Group D rotavirus infections in pheasants produced severe clinical disease (208).

In vitro propagation of rotavirus is successful when virus is trypsin activated and trypsin is also incorporated into the maintenance medium (163). These techniques have led to the successful isolation and propagation of group A rotaviruses from chickens (154, 155, 157), turkeys (154, 155, 157), pheasants (286) and ducks (251). A variety of primary (kidney and liver) and established (fetal rhesus monkey kidney cells, MA104 and Madin-Darby bovine kidney, MDBK cells) cells have been successfully utilized to propagate avian group A rotaviruses (116, 167, 254). Group D rotaviruses of avian origin have not been successfully isolated and/or propagated *in vitro*. A rotavirus isolated from lovebirds was lethal for chicken embryos when inoculated by the yolk sac route. The embryo mortality occurred 4-6 days following inoculation (85).

Enteroviruses and enterovirus-like viruses. Enteroviruses comprise a genus in the family *Picornaviridae* and include some of the important human (poliovirus) and mammalian (foot and mouth disease virus) pathogens. Single stranded RNA genome, round featureless morphology (under EM), non-enveloped, size of 28 nm, and resistance to chloroform and pH (pH 3.0) are some of the features on the basis of which primary identification is commonly made (202) . The avian pathogen included in this group are avian encephalomyelitis (AE) virus (24), avian nephritis (AN) virus (110), and various enterovirus-like particles that have been associated with chicken malabsorption syndrome (43, 45, 147, 159, 161, 165, 243) and poult enteritis (93, 152).

The physicochemical properties of the members of the enterovirus genus are generally used in identification as mentioned above. The enteroviruses which have been isolated from diarrheic chickens and turkeys, have a density of 1.33 g/ml in cesium chloride (93). The viral genome is composed of RNA and is approximately 7.5 Kb (93, 99). A cytopathic avian enterovirus isolated from chickens was resistance to chloroform, trypsin and acidic pH and was partially heat stable in the presence of 1M magnesium chloride (252). The avian enteric enteroviruses have been isolated and propagated with varying success in primary cells from chicken kidney and liver (162, 252). Embryonated chicken eggs have been used for virus isolation when injected via yolk sac and chorioallontoic membrane routes (93, 151, 161, 162, 243). One isolate from guinea fowl (cause of guinea fowl transmissible gastroenteritis) was successfully propagated in primary embryonic guinea fowl brain cell culture (186). The serologic characterization of avian enteroviruses has led to their classification into six groups on the basis of immunofluorescence test (46).

The association of enterovirus-like viruses with chicken malabsorption syndrome has been reported in Europe (43, 45, 147, 159, 161, 165, 243), Japan (252), and US (204, 205). In the UK, enterovirus-like particles were observed in the feces of 1- to 10-day-old normal chickens as well as chickens with malabsorption syndrome (147). The infection was mixed with rotaviruses, adenoviruses and reoviruses (44, 147). The situation was similar in the US where AN virus and reoviruses were observed along with enterovirus-like viruses in feces from runting and stunting syndrome affected chicks (79). The enteroviruses have been observed in the feces, pancreas and cecal contents of chickens with SS (44, 161, 204, 220, 252) as well as from meconium of dead-in-shell embryos (243). Although AE virus is transmitted vertically, the vertical mode of transmission for enteroviruses, presumably the cause of SS has not been investigated.

The incidence and prevalence of enteroviruses in turkey poults has been reported in the US from diarrheic (204, 219), healthy (147) as well as SPF (97, 208) turkey poults. The intestinal changes associated with enterovirus infections in chickens peak by 7 DPI and by 14 DPI, the intestines appear normal (45). In chickens there appears to be an age susceptibility to the enterovirus infection. Day-old chicks are more susceptible to infection than week-old-birds contrary to infection in turkeys where poults inoculated with enterovirus alone at 2-to-3-weeks of age are more severely affected (98).

Enteroviruses multiply in the cytoplasm of intestinal villus epithelium especially those at the base of the villi (47, 159). Histologically, there is variable shortening and blunting of villi, decreased villus-to-crypt ratio, increased crypt depth, desquamation of the intestinal epithelium, vacuolar degeneration of the enterocytes and mononuclear cellular infiltration of the lamina propria (45). There is glandular necrosis and lymphoid cellular infiltration of the proventriculus and pancreas (45).

Studies on the pathogenicity of enteroviruses in turkey poults have been limited. In one study poults infected (either orally or through contact exposure) with a "likely" enterovirus, developed clinical signs (watery droppings, dilated thin wall ceca filled with yellow foamy fluid, catarrhal enteritis and pale intestinal serosa), and gained less weight (249). Histologically, there was mild shortening of the duodenal villi and elongation of the crypts in the duodenum and ileum. The reported size of the enterovirus used in this study was 18-24 nm, smaller than the size of chicken enterovirus (27± 3 nm). Experimental mixed infection (combination of enterovirus and group A rotavirus) of SPF turkey poults resulted in more severe infection than poults inoculated with either inoculum alone (98). The parameters evaluated during this study were clinical signs, weight gain, D-xylose absorption, and intestinal morphometric analysis. Based on these observations it was also observed that

poults inoculated with enterovirus alone at 2- and 3-weeks of age were more severely affected than those inoculated at 3-days of age.

Reoviruses. Reoviruses are members of the genus *orthoreovirus* in the family *Reoviridae*. They are ubiquitous and occur worldwide. They infect a wide range of species including birds, cattle, humans, monkeys, sheep, mice, swine, etc. There are at least two antigenically recognized groups of orthoreoviruses. One group infects mammals and the other poultry. Avian orthoreoviruses do not infect mammalian species (64).

The reoviruses are icosahedral in structure and are non-enveloped. The virion possesses a double-shelled capsid which ranges in size from 60-75 nm. However, when the outer capsid is removed, the virus measures 60 nm in diameter. The diameter of the central compartment is 49 nm and holds 10 segments of double stranded RNA genome. Reoviruses have a buoyant density of 1.36-1.37 g/ml in cesium chloride. They are resistant to heat, chloroform, ether and hydrogen peroxide (244). Although commonly used disinfectants are ineffective against reoviruses, the viruses can be rapidly inactivated by 70% alcohol and 0.5% organic iodine (182).

The avian orthoreoviruses group has been further subdivided into 11 serotypes on the basis of neutralization tests (277). There is also a substantial cross neutralization between various types (277). Other studies, that addressed grouping reoviruses of chicken origin on the basis of serologic relatedness, have reported variable results (102, 213, 218). It was concluded from these studies that reoviruses from chicks commonly occur as antigenic subtypes rather than distinguishable serotypes (215). Reoviruses isolated from turkeys have been compared to chicken isolates. It was found that one turkey isolate (Georgia) was related to a chicken reovirus (Fahey-Crawely strain) (193) but was antigenically different from three

mammalian reoviruses tested (173). Serologic relatedness among turkey reoviruses has not been reported.

Reoviruses are present worldwide in chickens, turkeys, and other avian species (215). The outcome of reoviral infections in poultry range from inapparent to lethal disease. In chickens, the reoviruses have been found in association with arthritis, tenosynovitis, respiratory ailments, enteric disorders and malabsorption syndrome along with ruptured gastrocnemius tendons, myocarditis, pericarditis, hydropericardium, cloacal pasting and early chick mortality (215). However, they have been routinely isolated from the digestive tracts of normal and healthy chickens and turkeys (120) . Reoviruses can be vertically transmitted via the egg (164, 267). Therefore, it is very difficult to ascertain the true prevalence of reoviruses as an avian enteropathogen.

The isolation and propagation of avian reoviruses have been very successful using embryonating eggs following inoculation via the yolk sac or chorioallantoic membrane (CAM) routes (215). The yolk sac route is generally preferred. Reovirus inoculated embryos exhibit a purplish discoloration due to massive subcutaneous hemorrhage and die within 3-5 days. The embryos inoculated via the CAM are slightly dwarfed with occasional enlargement of the liver and spleen. The mortality occurs between 7-8 days post inoculation. Reoviruses grow in primary cell cultures of embryonic lung, kidney, liver, testicles and macrophages (11, 91). Chicken embryo fibroblasts can also be used for reovirus propagation but usually requires adaptation by the virus. Infected cells form syncytia followed by degeneration with eosinophilic or basophilic intracytoplasmic inclusions. Among various cell lines, reoviruses have been successfully isolated in baby hamster kidney (BHK) 211/13, feline kidney (CRFK), Georgia bovine kidney (GBK), rabbit kidney (RK) and porcine kidney (PK) cells (11).

Reoviruses have been frequently isolated from chickens experiencing runting/stunting syndrome (80, 184). However, to date, the cause-and-effect relationship of reoviruses and SS has not been well established in chickens (43, 129). The reoviruses vary in their pathogenicity for poults (70, 75, 76, 177, 240, 282) as an enteropathogen.

Togaviruses and togavirus-like viruses. The members of the family *Togaviridae* have been grouped into two genera; *alphaviruses* and *rubiviruses*. In a more recent classification system, pestiviruses and other viruses which are not well characterized and originally were the members of togavirus groups have been placed in the family *Flaviviridae*. The *alphaviruses* comprise a group of 27 different members which clearly differ in their ability to cause disease. The togaviruses consist of a single strand of positive sense RNA which is encapsidated in a icosahedral protein shell composed of a single species of protein. The viral envelope, studded with viral encoded glycoproteins, is derived from the host cell plasma membrane (226).

The members of *alphaviruses* that have been found to be associated with diseases in domestic poultry include eastern equine encephalitis virus and western equine encephalitis virus. The turkey meningoencephalitis virus has been placed in the family *Flaviviridae* (108, 109).

The togaviruses and/or togavirus-like viruses have also been incriminated as a cause of stunting syndrome in chickens. In 1986, togavirus-like particles were observed in the portions of ventral pancreatic duct from 7-11 day old chickens with a history of SS (66). Similarly togavirus-like particles have also been demonstrated in jejunal enterocytes and intestinal contents of chickens experiencing infectious stunting syndrome (66). Togavirus-like particles have also been observed in primary cultured fibroblasts cells from SPF chicken embryos (61, 68, 77). These viruses caused only minimal (68) or no cytopathic effects (61). The primary embryo

fibroblast cell cultures, therefore, would be of limited use for identifying these viruses.

The identification of the particles as togavirus-like has been based upon their mode of replication and morphology when examined ultrastructurally *in situ*. Although no further attempts have been made to identify and/or classify these particles, their exact nature remains a mystery. Moreover, there are no reports on experimental reproduction of any disease using a togavirus-like agent. The presence of togavirus-like particles in the chick embryo fibroblasts may also suggests the possibility of vertical transmission.

Togavirus-like particles have been found to be the cause of a lethal outbreak of a fulminating disease in guinea fowl chicks (19). The isolated virus had a morphology consistent with that of togavirus-like agent when observed under EM, a buoyant density of 1.18 g/ml in sucrose and hemagglutinated goose erythrocytes. Inoculation of day-old guinea fowl poults with purified preparations caused anorexia and emaciation. At necropsy, there was loss of intestinal tone, distended ceca with yellow foamy material, sever watery diarrhea, pancreatic necrosis, distention of gallbladder and swollen kidneys. Eastern equine encephalitis (EEE) virus and Highlands J virus have been found in two commercial turkey flocks experiencing watery diarrhea and typical EEE symptoms (restlessness, somnolence and sudden death (63). But at the same time reovirus and picornavirus particles were also detected from the feces of these birds.

Arena-like virus particles have been associated with hypoglycemia, enteritis, and spiking mortality (37)

Calicivirus. The family *Caliciviridae* include Norwalk and the Norwalk group of viruses, which cause mild, self limiting gastroenteritis in humans (119). Enteric calici- and calici-like viruses are about 30-40 nm in diameter. When examined by EM, the surface of calicivirus have dark spherical depression of 10 nm in diameter.

These depression are formed by the deposits of the negative stain filling the cup-shaped depression on the virus surface (hence the name calici- from the Latin word "calyx" meaning cup) (247). There is limited information regarding the physico-chemical properties of caliciviruses. This is primarily because of the inability to grow these viruses in cell culture or in organ culture (50, 119). The virus has a positive sense polyadenylated single stranded RNA genome. Studies using human volunteers for virus propagation have shown that the virus has a density of 1.36 - 1.41 g/cm^3 in cesium chloride, is stable at 60 C for 30 minutes, is resistant to pH 2.7 for 3 h at room temperature and resists 20% ether at 4 C for 18 hours (50).

Calicivirus, on the basis of EM morphology, have been demonstrated in the intestines of 4-week-old chickens with a history of infectious stunting syndrome. The virus could not be grown *in vitro* (283). Calicivirus-like particles were found in 6-to-16-day-old guinea fowls (*Numida meleagris*) with nervous signs and 50% mortality (81). There is one report about successful propagation (in primary chick embryo fibroblasts) of calicivirus-like virus particles obtained from the gut homogenate of stunted chickens (33). The morphological and biophysical properties were similar to those of feline caliciviruses (33). The avian calicivirus has a RNA genome and replicates in the presence of actinomycin D. The buoyant density is 1.38-1.39 g/ml in cesium chloride. Caliciviruses have been detected from the feces and intestinal contents of 3- and 4-week-old pheasants with low grade enteritis, weight loss and paralysis (86). Experimental infection of day-old chickens with calicivirus led to loose droppings, failure to thrive, and death (33). The virus was demonstrated from feces of experimentally infected chicks, however, their exact role could not be ascertained as the inoculum was contaminated with reoviruses.

FEW virus. Spherical particles of 45-55 nm in diameter, which were termed FEW virus, were isolated from the gut homogenates of 4-day-old chicks with early signs of infectious stunting (60). The virus had a RNA genome. It was stable at pH

3.0 and resistant to treatment with trypsin. The nature of the FEW virus was not identified as it was not neutralized by the antiserum to infectious bronchitis virus, reticuloendotheliosis virus, avian encephalomyelitis virus, avian influenza virus, infectious bursal disease virus, rotavirus and reovirus.

Parvoviruses. Small viral particles with a diameter of 19-24 nm and a buoyant density of 1.43 g/ml in cesium chloride were observed in the intestines of 10-day-old broiler chickens with stunting syndrome (113). These particles were later confirmed as parvovirus on the basis of genome characteristic and were classified as fowl parvovirus type 1 (ABU strain) (114). Experimental inoculation of day-old SPF chickens had very little effect on the growth rate. But day-old commercial broiler chickens inoculated with the ABU strain of parvovirus showed growth retardation, poor feathering and soft bones (115). The inoculation of embryos with the ABU parvovirus led to poor hatchability and reduced livability (115). The virus was detected by the immunofluorescence technique in the epithelium of the small intestine. In contrast, clinical signs and growth retardation effects could not be reproduced in another study when day-old SPF or commercial broiler chickens were inoculated with a preparation of partially purified ABU parvovirus (165). The virus was detected in the feces of infected birds after 7 days post inoculation. Parvovirus-like particles were observed in turkeys experiencing stunting and diarrhea (262)

Non-avian viruses.

Toroviruses. Torovirus is a newly recognized genus in the family *Coronaviridae*. Toroviruses have been given the name because of the characteristic morphology of its members (Latin word 'torous' means toroid shape). When examined by EM, the torovirus appears as elongated- (straight nucleocapsid), kidney- (C-shaped nucleocapsid), or spherical/doughnut/erythrocyte-shaped (with nucleocapsid as O-ring). Thus, the torovirus are pleomorphic and range in diameter

from 30-120 nm for kidney shaped particles and 75-90 nm for circular particles (125).

Information concerning toroviruses is relatively recent and brief. Berne virus, the first representative and the type species of the genus, was isolated from rectal swabs taken from a horse with diarrhea (271). Subsequently, morphologically similar particles have been observed in different species including humans (13). Bovine enteric toroviruses termed Breda viruses (BRV) have been found in association with diarrhea in calves and have produced disease in gnotobiotic calves under experimental conditions (279, 280). More recently, bovine toroviruses have been isolated (268) from the respiratory tracts of calves (BRTV; bovine respiratory torovirus). Toroviruses and torovirus-like particles have been found in different animal species such as dogs (65), cats (172) and swine (227). Antibodies to toroviruses are even more wide spread as these have been detected in sheep, goats, rabbits, laboratory mice, etc. (272).

Among all the species of torovirus, only the Berne virus has been successively propagated in equine dermis or embryonic mule skin cells (271). The CPE includes cell lysis. Virus treated with trypsin or ß-chymotrypsin results in a marked increase in infectivity (273). Repeated attempts to isolate additional equine strains of toroviruses have not been successful. Efforts to isolate Breda viruses *in vitro* using Madin-Darby bovine kidney cells (MDBK) and organ culture of intestinal tissue have been unrewarding (279). Similarly, toroviruses from humans, cats and pigs have not been propagated *in vitro*.

The physicochemical properties of toroviruses have only been described for the Berne virus. They have a density of 1.16 g/ml in sucrose gradients (271) and are sensitive to chloroform and diethyl ether (273). However, the viral infectivity is only marginally influenced by treatment with phospholipase C or sodium deoxycholate. The virus is stable over a wide range of pH (pH 2.5 to 10) (273). The virus has a

RNA genome (271). Bovine Breda virus hemagglutinates red cells from rats (125, 279) while Berne virus has a hemagglutinin for human blood group O erythrocytes (294).

Breda virus infections probably spread by the fecal oral route. The stability of the virus in the environment has not been fully explored however, the carrier animals have been proposed to be a source of virus.

Bacteria.

The role of bacteria in the causation, development and progression of SS has not been extensively explored. Bacteria-free filtrates have been shown to elicit some aspects of the syndrome but not the whole disease syndrome (140, 231). *Campylobacter jejuni* isolated from stunted chickens was unable to reproduce the disease when inoculated into susceptible chicks (17, 18). Long segmented filamentous organisms (LSFOs) have been observed in poults with SS (4). Long segmented filamentous organisms have been observed in normal mice, rats and chickens (26, 36, 71, 94). These organisms are attached to the intestinal epithelial cells at the tip of the villus and are seldom found below the top third of the villus length. The head of the filament is attached to the apical border of the epithelial cell membrane causing a depression in the apical plasma membrane. The penetration of the epithelial cell was not evident in all studies. The microvilli at the site of attachment were either displaced, absent or modified. The inflammatory response was completely absent. The LSFOs were not observed within the epithelial cells. The LSFOs have not been cultivated *in vitro*. The role of these inhabitants in the intestines is not known but has been proposed to stimulate the immune response in some animals (222). There is one report of the *in vitro* isolation of LSFOs but their morphology appears different from the known LSFOs (171).

Others.

Enteric parasites such as cryptosporidium have been observed in poults with diarrhea but their exact role has not been established (78). Other non-infectious yet unknown factors might play a role(s) in SS. These factors might include toxin(s), nutritional deficiencies and/or combination of virus(es), bacteria and other factors.

CHAPTER 3. PRIMARY CELL CULTURE OF TURKEY INTESTINAL EPITHELIAL CELLS

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Summary. Primary cell culture has been widely used for various types of studies and proven useful for the isolation and identification of avian pathogens. Difficulties in growing intestinal epithelial cells *in vitro* has limited their use for such studies. In the present study, a co-culture system was developed for the primary culture of intestinal epithelial cells. A monolayer obtained from 14-to-16-day-old turkey embryo intestinal fibroblasts was used as a feeder layer. Feeder layers from turkey embryo fibroblasts and from a continuous cell line (mouse 3T3 fibroblasts) were also employed but were not as successful. The intestinal epithelial cells were isolated by dissociation from the intestinal tracts of 1-day-old turkey poults and grown on the feeder layers. Growth and maintenance media were supplemented with various components including fetal calf serum, chicken serum, hormones and other growth factors. The epithelial cells grown on feeder layers from the intestinal fibroblasts allowed the intestinal epithelial cells to be maintained *in vitro* for periods of 7-10 days. This technique may prove useful for various applications including isolation of enteropathogens and basic studies of the intestinal tract concerning such subjects as physiology, immunology, and toxicology.

Key Words: Intestinal cell culture; Intestinal epithelial cells; Intestinal fibroblasts feeder layer.

Abbreviations: BSA, bovine serum albumin; CMF-PBS, calcium-magnesium free phosphate buffer saline; DNase, deoxyribonuclease; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; HEPES, (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]); PBS, phosphate buffer saline.

INTRODUCTION

Primary culture of animal cells and tissues is a widely used technique in the field of medicine and cellular and molecular biology. These systems have been employed for various applications including isolation of avian viral pathogens. Various types of animal cells have been cultured successfully *in vitro* including fibrocytes, kidney cells, hepatic cells, and lung cells (6). The difficulties encountered in growing intestinal epithelial cells *in vitro* have limited their applications. To date, none of the continuous cell lines obtained from normal undifferentiated cells have allowed the generation of fully functional and differentiated enterocytes (10). Several attempts have been made to culture intestinal cells from adult (14,20), postnatal (4,16,22) and fetal (12,13) animal intestines. Among these techniques, organ culture and presumably epithelial-mesenchymal co-culture systems have been considered the most successful.

The mechanisms by which intestinal epithelial cells proliferate and differentiate are considerably complex and largely unknown owing to the cellular heterogeneity of the intestinal epithelium, and to the constant and rapid turn over of epithelial cells. A number of other factors have been found to play a role in the regulation of intestinal epithelial cell proliferation and differentiation. These include components of extracellular matrix (3), mesenchymal-parenchymal interactions (11), polyamines (15) and hormones (9). Of these, the mesenchymal-parenchymal interactions are of major importance. These interactions presumably are responsible for the synthesis of basement membrane matrix products [type IV collagen, laminin, nidogen/entactin, heparan sulphate proteoglycan, fibronectin and tenacin, (5, 7, 18)] that have been postulated to participate in epithelial cell adhesion, proliferation, and differentiation (1,8). These factors illustrate the complex role of epithelial-mesenchymal interaction in normal gut development.

The objective of the present study was to establish a technique to culture intestinal epithelial cells *in vitro*. One homologous feeder layer (turkey embryo intetstinal fibroblasts) and two heterologous feeder layers (turkey embryo fibroblasts and mouse 3T3 fibroblast continuous cell line) were evaluated as co-culture with poults intestinal epithelial cells. Two protocols were evaluated for the preparation of embryonic intestinal fibroblast feeder layers. Additionally, the prepared substrate mixtures of collagen type IV, collagen type I, laminin, fibronectin, bovine serum albumin and heparan sulfate were tested for their ability to support the growth of epithelial cells. Basement membrane preparations from turkey embryo fibroblasts, mouse 3T3 fibroblasts and intestinal fibroblasts were also evaluated for epithelial cell attachment and growth.

MATERIALS AND METHODS

Intestinal fibroblast feeder layers. The feeder layers were prepared from turkey embryo intestinal fibroblasts using two different protocols. All reagents were obtained from Sigma Chemical Co. (St. Louis, Missouri) unless otherwise indicated.

Protocol I. The intestines (from the proximal duodenum to distal ileum) were collected from 14-16-day-old commercial turkey embryos and cut into small (2-3 mm) pieces. The pieces were washed several times in Dulbecco's modified Eagle's medium (Life Technologies Inc., Gaithersburg, Md) containing 5% fetal calf serum (FCS; JRH Biosciences, Lenexa, Kan.), 2 µg insulin/ml, 25 mM HEPES, 2 g glucose/l, and 25 µg gentamicin/ml (working medium). The pieces were pre-incubated in this medium for 15 min at 37 C with mild stirring. Following pre-incubation, the pieces were digested for 3-4 h in the following enzyme mixture: 5 mg collagenase type I/ml, 5 mg hyaluronidase type I-S/ml and 0.5 mg DNase/ml in the working medium. The resultant cell mixture was filtered through 4-6 layers of sterile cheesecloth. The isolated cells were washed several times with 40 ml of the working medium and each wash consisted of resuspending cells followed by

centrifugation at 400 x *g* for 8 minutes. The cell concentration was adjusted to 5 x 10^5 cells/ml and dispensed in minimum volume (8-10 ml) to cover the surface of the plastic tissue-culture flasks (75 cm^2 T-flasks; Corning Inc., Corning, N. Y.). The flasks were incubated for 3-4 h in working medium at 37 C with 5% CO_2 in a humidified incubator. The unattached cells were decanted, and fresh medium was added every third day until the monolayer was about 50% confluent.

Protocol II. The intestines were collected, cut into small pieces, and washed as described above. The surface of a 75 cm^2 plastic tissue culture flask was roughened by being scratched gently with a scalpel and then washed with medium several times. The tissue pieces (approximately 50 pieces per flask) were added to the flask in an amount (4 - 5 ml) of working medium (with 50% FCS) small enough to prevent them from floating. Following overnight incubation at 37 C in 5% CO_2, the medium volume was increased to 30 ml with working medium supplemented with 5% FCS and incubation was continued for an additional 24 - 48 hr. Under these conditions, the only cells observed to have grown out from the explants were fibroblast-like cells. All the tissue pieces were then removed using a fine sterile forceps. The flasks were incubated until the monolayer was about 80% confluent (5-6 days) and subcultures were obtained by trypsinization (0.025% trypsin and 0.02% ethylenediaminetetraacetic acid [EDTA] in calcium-magnesium free phosphate buffered saline, CMF-PBS, pH 7.0). The fibroblasts were used at passage 2 or 3, and a 40-50% confluent monolayer was used for co-culturing.

Mouse 3T3 fibroblast feeder layer. Feeder layers derived from a continuous cell line of mouse 3T3 fibroblasts (ATCC cat # CCL 92) was prepared following a published protocol (21). Briefly, the cells were grown in DMEM supplemented with 10% bovine calf serum; 24 hr. after the monolayer reached confluency, it was exposed to mitomycin C (10 µg/ml) for 2 hr. The monolayer was washed four times with PBS (pH 7.2), trypsinized, and the cells were dispensed into plates at a density

of 3 x 10^6 cells/ml. The cells were allowed to adhere to the tissue-culture plates for 24-48 hr before they were used as feeder layers.

Feeder layer from turkey embryo fibroblasts. Whole embryo fibroblasts were prepared from 10-day-old turkey embryos using techniques described for preparing chicken embryo fibroblasts (17). The cells were grown in DMEM supplemented with 8% FCS and were used following 2 or 3 passages. The subcultures were obtained by trypsinization as described above.

Isolation of intestinal epithelial cells. On the day of hatch, 8 to 10 poults were euthanized with CO_2, and the entire small intestines were removed. The attached mesentry was removed and intestines were cut longitudinally and then into 2-to-3-cm-long pieces. To eliminate mucus, the pieces were gently swirled, and then incubated with N-acetyl cysteine (0.15% in medium) for 20 minutes at room temperature. The epithelial cells were dissociated by incubating the intestinal pieces in medium (Joklik's modified minimum Essential medium [MEM] supplemented with 25 mM, 2.5 g glucose/l, 1% BSA, and 50 µg gentamicin/ml [pH 5.5] prewarmed to 37 C) containing 1 mM EDTA at room temperature, with gentle shaking. Those cells dissociated during the first 5 minutes of incubation were discarded, fresh medium was added, and incubation was continued for another 25 minutes. The dissociated cells were decanted and washed several times to eliminate residual EDTA. The pellet from the final washing was resuspended in 30% Percoll® (Pharmacia Biotech Inc., Piscataway, N. J.) and layered on top of 60% Percoll® (pH 5.5). The mixture was centrifuged at 500 x *g* for 20 minutes at room temperature and cells collected from the top layer (epithelial cells) were washed 5 or 6 times. After the final washing, the cells were counted and the concentration was adjusted to 4 x 10^6 cells/ml in growth medium. The growth medium consisted of a basal medium, either CMRL-1066 or DMEM, supplemented with 25 mM HEPES, 5% FCS, 5% chicken serum, penicillin-streptomycin-fungizone mixture (100 U penicillin/ml, 100 µg

streptomycin/ml, and 0.25 µg fungizone/ml; JRH Biosciences, Lenexa, KS), 2 g glucose/l, 38 µg ascorbic acid/ml, 1.5 µg transferrin/ml, 4 mM L-glutamine, 10^{-8} M sodium selenite, 20 ng epidermal growth factor/ml, 5 µg pentagastrin/ml, 10^{-8} M desoxycholic acid, 0.2 M progesterone, 25 ng triiodothyronine/ml, 10 µg/ml insulin and 5 µM putrescine (pH 6.9).

Co-culture of epithelial cells on feeder layer. Primary fibroblastic feeder layers were used when they reached 40-50% confluency. The growth medium was removed from the feeder-cell monolayer, and epithelial cells suspended in growth medium (4×10^6 cells/ml) were added (25 ml/75 cm^2 T-flask) centrifuged onto feeder layers (150 x *g* for 8 min. at room temp). The co-cultures were incubated at 37 C in 5% CO_2 in a humidified incubator. Half the volume of the growth medium was replenished with fresh growth medium on alternating days and pH was monitored ciosely during incubation to maintain a range of 6.8-7.0. The viability of epithelial cells was evaluated on alternating days by means of trypan blue exclusion, and the percentage of live cells was determined.

Culture of epithelial cells on prepared substrate. The culture plates (six-well plastic tisssue-culture plates; Corning Inc.) were coated with BSA (0.5 µg/ml), fibronectin (2 µg/cm^2), collagen IV (5 µg/cm^2), laminin (1 µg/cm^2), collagen I (5 µg/cm^2) and heparan sulfate (5 µg/cm^2) in MEM by incubating the dishes at 37 C in 5% CO_2 for 8-24 h in a humidified incubator. Before the addition of cells the medium was carefully aspirated, leaving a thin film behind. Epithelial cells were then centrifuged onto the substrate as described above and the growth medium was changed on alternating days. Again the viability of epithelial cells was tested by trypan blue exclusion, and the percentage of live cells was calculated.

Basement membrane preparation. Fibroblast cultures were prepared from whole turkey embryos, turkey embryonal intestine or mouse fibroblast cell line (3T3); the cells were seeded at half the concentration used for normal seeding. When the

monolayers were nearly 100% confluent, they were washed three times with calcium magnesiu-free PBS (pH 7.2) and exposed to 0.025 M NH_4OH. The resulting attached membranes were washed several times with PBS and allowed to air-dry under sterile conditions in a laminar flow hood. Just before use, the dishes were moistened with 1 ml of growth medium, and the epithelial cells were centrifuged onto the membrane preparation. Fresh growth medium was added on alternating dayst; the viability of epithelial cells was determined by trypan blue exclusion, and the percentage of the viable cells was counted.

RESULTS

The epithelial cells viability was greater than 85% after 8 days when cells were co-cultured in the presence of the homologous fibroblastic feeder layer using protocol I (Fig. 1). The viability of epithelial cells declined to about 65% after 6 days when protocol II was used. Epithelial cells cultured without a feeder layer were less tha 40% viable after 4 days of incubation (Fig. 1). The intestinal fibroblast feeder monolayer derived from protocol I is shown in fig. 2. The epithelial cells co-cultured with homologous fibroblasts appeared to be granular morphologically with some cells showing a typical columnar appearance (Fig. 3). A 40-50% confluent homologous feeder monolayer used in co-culturing the epithelial cells was found to be better than a confluent monolayer, because the cells tended to detach during incubation when 100% confluent monolayers were used. Epithelial cells cultured with fibroblasts derived from turkey embryos, cultured with mouse 3T3 fibroblasts continuous cell line or cultured without feeder layers did not do well after 4 days (Fig. 4). The viability of the epithelial cells declined to less than 50% after 4 days. This was also observed when epithelial cells were cultured on basement membranes prepared from fibroblasts or formulated substrates. The viability declined rapidly after three days and only a few cells (less than 25%) were alive after four days (Fig. 5).

DISCUSSION

The primary culture of intestinal epithelial cells has been problematic because of their inability to be maintained by conventional cell culture methods. However, the role that the basement membrane and the underlying fibroblasts play in maintaining the epithelial integrity and cell renewal *in vivo* has provided some insight which has aided the *in vitro* support and growth of the intestinal epithelial cells. For example, the intestinal fibroblasts obtained from a human fetus, when co-cultured with Caco-2/15 cells, produced all the constituents of a basement membrane (19). In the present study, the intestinal epithelial cells were co-cultured in the presence of intestinal fibroblasts and survived longer than those cells cultured without fibroblasts. The homologous co-culture system (intestinal fibroblasts and

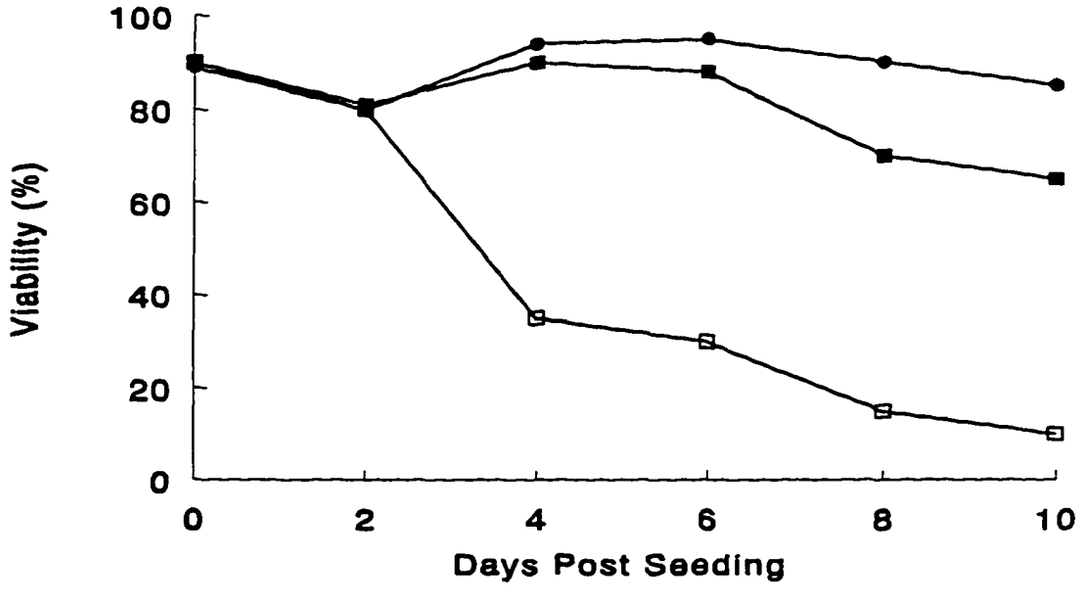

Fig 1. Viability of intestinal epithelial cells over time when cells were cultured with homologous fibroblast feeder layers prepared by protocol I (●), protocol II (■) or no feeder layer (□).

Fig 2. Intestinal fibroblast feeder monolayer (40-50% confluent) obtained from 14-16-day-old turkey embryos. The cells show typical fibroblast morphology.

Fig 3. Intestinal epithelial cells co-cultured with an intestinal fibroblast feeder monolayer. The scattered fibroblasts (a) cells are visible. The epithelial cells appear as granular cells with variable morphology (b). Some cells show columnar appearance (c). (x200).

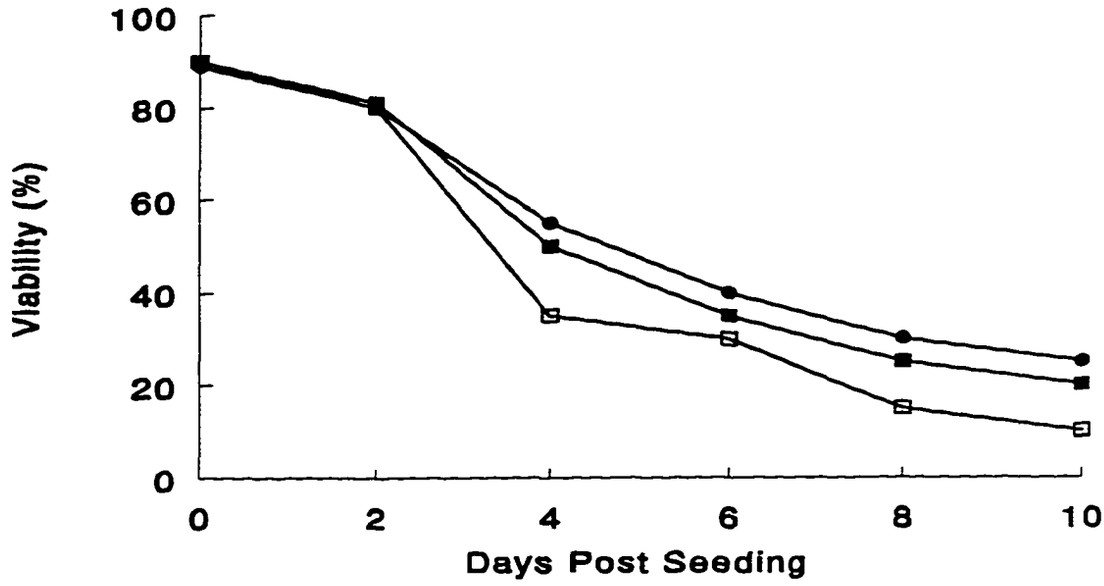

Fig 4. Viability of intestinal epithelial cells over time when cells were cultured with heterologous fibroblast feeder layers prepared from primary embryo fibroblast (●), the 3T3 fibroblast continuous cell line (■) or no feeder layer (□).

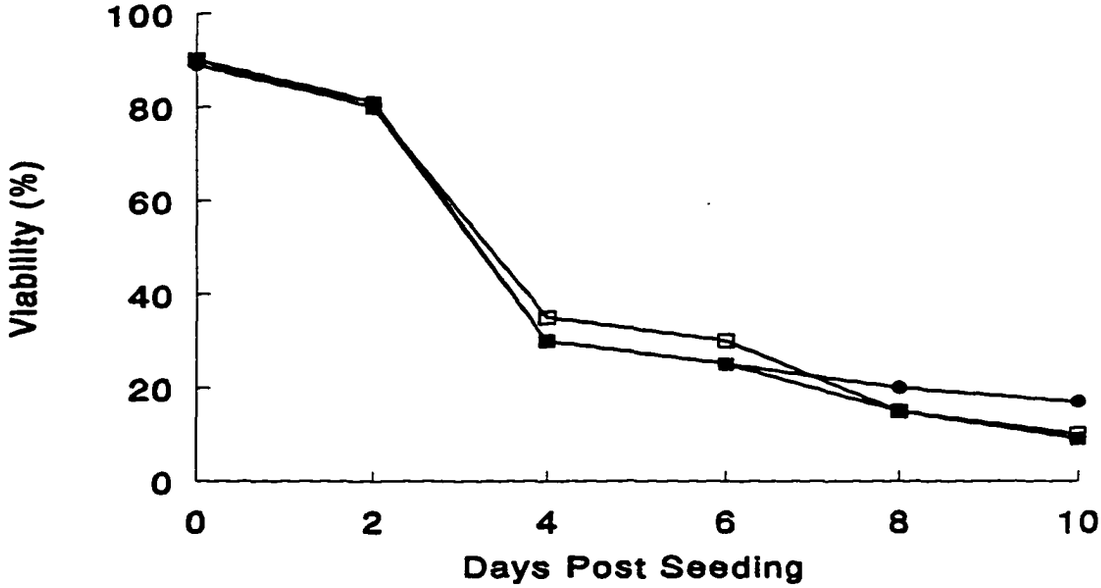

Fig 5. Viability of intestinal epithelial cells cultured without a feeder layer using a prepared substrate (●), a basement membrane preparation (■) or in the absence of these materials (□).

intestinal epithelial cells) proved to be better than the heterologous system. This has also been noted in similar studies in which the intestinal fibroblasts stimulated growth of intestinal epithelial cells whereas fibroblasts obtained from skin did not stimulate growth (5,7).

A combination of basement membrane constituents and prepared substrates did not support the epithelial cell survival in present study. Similar results have been reported for human colon epithelial cells (2). Cells were not supported by this method possibly because of variation in concentration and composition of the constituents and/or provision of some additional matrix and/or growth factors by the fibroblasts. A pure population of intestinal fibroblasts appeared to be less supportive than a mixed population of cells from the intestine. Based on this observations, there may be other cells in the intestine that aid epithelial cell growth. Other factors found to be important were centrifugation of epithelial cells onto the feeder layer and slightly acidic (pH 6.9) growth media.

The *in vitro* intestinal epithelial cell-culture methodology using homologous fibroblast feeder layer may be useful in studying the basic physiology of intestinal epithelial cells and may also benefit investigators involved in other areas such as nutrition, toxicology, etc. Additionally, this technique may be valuable for the isolation, propagation and identification of infectious agents, particularly fastidious enteric pathogens.

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CHAPTER 4. STUNTING SYNDROME IN TURKEY POULTS: ISOLATION AND IDENTIFICATION OF THE ETIOLOGIC AGENT

A paper accepted by Avian Diseases

Akbar Ali and Donald L. Reynolds

Summary. Stunting syndrome (SS) is an enteric disease of turkey poults that causes high morbidity including reduced growth, impaired feed efficiency and diarrhea. The etiologic agent of this disease has not been previously reported. The objectives of the present study were to identify, isolate and purify the etiologic agent of SS. Day-old poults were orally inoculated with a SS inducing inoculum. The intestinal epithelial cells (IECs) were isolated on the fourth day postinoculation. The IECs were lysed and filtered through 0.2-, 0.1-, and 0.02-μm filters. The cell lysate filtrate (0.1 μm) was subjected to density gradient ultracentrifugation. Intact IECs, filtrates from IECs (0.2, 0.1, 0.02 μm), and IEC lysate fractions from gradients (FRG) were used as inocula to infect day-old turkey poults. The weight gain, jejunal maltase activity and gross intestinal lesions were used as the parameters of evaluation. Weight gain and maltase activity were reduced ($P \leq 0.001$) by the isolated IECs, 0.2 and 0.1 μm filtrates, and FRG when compared to corresponding controls. IEC lysate filtrate (0.1 μm) and FRG were examined under transmission electron microscope (EM). Enveloped, pleomorphic particles varying in size from 60 to 95 nm were observed and termed stunting syndrome agent (SSA). Primary cultures of turkey IECs were used to further isolate and propagate the SSA. Following the fifth passage in the turkey IECs, the cell lysate induced SS in day-old poults. SSA particles were observed under EM after the fifth passage. The results of this study provide evidence that a viral agent has been isolated and identified from IECs of poults inoculated with SS inoculum and is the etiologic agent of SS.

Key words. Stunting syndrome; intestinal epithelial cells; poult enteritis; turkey viral enteritis; enteric virus.

Abbreviations. BSA = bovine serum albumin, EDTA = ethylenediaminetetraacetic acid, EM = electron microscopy, FCS = fetal calf serum, FRG = fraction from gradient, IECs = intestinal epithelial cells, MEM = minimum essential medium, PBS = phosphate buffered saline, PI = post inoculation, PTA = phosphotungstic acid, SS = stunting syndrome, SSA = stunting syndrome agent, TNEM Buffer = 10 mM tris, 0.1 M NaCl, 2 mM $MgCl_2$, 0.02 M EDTA (pH 7.0), TPB = tryptose phosphate broth.

INTRODUCTION

An enteric condition in young turkeys, previously reported and referred to as stunting syndrome (SS), is commonly observed during the first few weeks after placement (4). The most common manifestations of SS include poor growth, decreased feed utilization, diarrhea, depression and enteritis (16, 21). The gastrointestinal tract appears to be a primary target organ. Typical necropsy findings include cecal dilatation with gaseous yellow to brown contents and pale, thin intestines with watery contents (16, 21, 24).

The etiologic agent of this disease has not been reported. A number of viral agents have been documented to cause enteric diseases in turkey poults (e.g. rotaviruses [17, 24, 26, 29], reoviruses [24, 28], coronaviruses [22, 24], enteroviruses [12, 26, 29], astroviruses [18, 23, 25, 26, 29], etc.). Some of these agents have been implicated as being involved in SS, but to-date, no definitive role for any of these agents has been established.

The contribution of bacteria in the causation, severity, and pathogenesis of SS is not well understood. It was previously reported that inoculation of day-old poults with a bacteria-free filtrate induced SS (32). Long segmented filamentous organisms have been observed in affected poults (3, 19) but their role in SS is

unknown. Since bacteria-free filtrates were shown to induce SS, a viral etiology seems plausible.

The objectives of the present study were to isolate and identify the etiologic agent of SS in turkey poults.

MATERIALS AND METHODS

Poults. Day-old commercial turkey poults were used throughout the studies. Poults were reared in positive-pressure, plastic bubble-type containment isolators equipped with intake and exhaust filters (Standard Safety Equipment Co., Palatine, IL). The isolator was approximately 1m wide, 0.7 m high and 2 m long. Contained within each isolator were plastic cages with stainless steel dropping pans. Feed and sterile water were provided *ad libitum*.

Reagents and chemicals. All reagents and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Stunting Syndrome inoculum. The original SS inoculum was obtained from Dr. Jerry Sell (Iowa State University, Ames IA) and was prepared according to a previously reported study (4) and served as positive SS inoculum. A negative SS inoculum was prepared by the same method as the SS inoculum (as above) except the intestines from SS-free poults were used. Tryptose phosphate broth (TPB) was used as the negative control inoculum.

Isolation and purification of intestinal epithelial cells (IECs). IECs were isolated and purified according to a described method (2). Briefly, the entire small intestines were removed and cut into 2-3-cm-long pieces. The pieces were washed in phosphate-buffered saline (PBS; pH 6.0) to rid them of digesta. The mucus was removed by incubating the intestinal pieces with 0.15% N-acetyl cysteine in wash medium (Joklik's modified minimum essential medium [MEM] with 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 2 g/liter dextrose, 1% bovine serum albumin [BSA], 2 ml/100 ml of penicillin-streptomycin-fungizone mixture [pH

5.5] pre-warmed to 37 C) at room temperature for 20 min. The IECs were dissociated by incubating the pieces with 1 mM ethylenediamine tetraacetic acid (EDTA) in wash medium at room temperature for 1 hr. The dissociated cells were purified on Percoll® (Pharmacia Biotech Inc., Piscataway, NJ) and washed several times with BSA-free wash medium. The total cell count was determined by using trypan blue dye exclusion.

Lysis of IECs. IECs were lysed using the Parr® 4635 cell disruption bomb (Parr Instrument Co., Moline, IL) following a previously described procedure (13). Cells (10^{10} cells/ml) were suspended in ice cold lysis buffer (0.0002 M $CaCl_2$; pH 6.0) and poured into the chilled vessel. The vessel was sealed, and nitrogen gas was introduced slowly until an equilibrium pressure of 1300 lb/in^2 was achieved. The vessel was placed on ice and cells were stirred under pressure for 25 min. The suspension was expelled (under pressure) into a long neck ice chilled flask. The mixture was centrifuged at 5000 x g for 30 min. The supernatant was saved, and the pellet was subjected to lysis again following the same procedure. The cell lysate was centrifuged again and the supernatant was adjusted to 10 mM tris, 0.1 M NaCl, 2 mM $MgCl_2$ and 0.02 M EDTA by using a 100x concentrated solution of the same.

Cell lysate filtration. The cell lysate was serially passed through filters of 5, 3, 0.8, 0.65, 0.45, 0.2, 0.1 and 0.02 µm pore size. Filtrates following 0.2-, 0.1-, and 0.02-µm pore size filtration were centrifuged at 200,000 x *g* for 4 hr at 4 C. The pellet was suspended in TPB.

Density gradient centrifugation. Following filtration through 0.1-µm filter, the IEC lysate was centrifuged at 200,000 x *g* for 4 hr. The pellet was subjected to density gradient centrifugation using CsCl, sucrose and Accudenz® (5-(2,3-dihydroxypropylacetamido-2,4,6-tri-iodo-N,N'-bis (2,3 dihydroxypropyl) isophthalamide; Accurate Chemical & Scientific Co., Westbury, NY). The CsCl isopycnic gradient was prepared by suspending the pellet in a CsCl solution (density

of 1.39 g/cm^3 in TNEM buffer [10 mM tris, 0.01 M NaCl, 2 mM $MgCl_2$ and 0.02 M EDTA; pH 7.0]) and centrifuging at 200,000 x *g* for 24 hr at 4 C. Sucrose and Accudenz® step gradients were made by successively layering 50, 40, 30, 20 and 10% sucrose and Accudenz® solutions (in TNEM buffer), respectively, to form a discontinuous step gradient. The pellet was suspended in TNEM buffer and layered above the discontinuous gradient. The gradients were centrifuged at 200,000 x *g* for 4 hr (sucrose) or 16 hr (Accudenz®) at 4 C. Following centrifugation, visible bands were collected and dialyzed against five or six changes of TNC (10 mM tris, 0.01 M NaCl, 20 mM $CaCl_2$; pH 7.0) buffer at 4 C. The density of each recovered band was determined from the refractive index measured at room temperature on a refractometer (Abbe 3L refractometer; Milton Roy, Rochester, NY).

Measurement of intestinal maltase activity. Poults were euthanatized and the jejuna were removed. The jejuna were flushed with ice-chilled PBS and the intestinal tissue was frozen immediately in liquid nitrogen. The jejunal tissue was prepared for further analysis as described previously (31), and the total protein was measured by the method of Lowry (15). Maltase activities were determined by the method of Dahlquist (7). The specific maltase activity was calculated as enzyme activity based on percent protein.

Electron microscopy. Samples were diluted appropriately with sterile distilled water and stained with an equal volume of phosphotungstic acid (PTA) solution (PTA 3%, sucrose 0.4%; pH 6.0) solution for 60 sec. The virus-PTA mixture was then applied to Formvar® (Electron Microscopy Science, Fort Washington, PA) carbon-coated 300-mesh copper grids. The grids were observed under a Hitachi-500 transmission electron microscope at 75 KV.

Experimental design. The specific protocols for each trial is given below. In trial I, II, III, IV and VI, there were two experiments per trial. In the first experiment, poults were inoculated orally, whereas in the second experiment, poults were

environmentally exposed to infection by placing them in the previously contaminated isolators that were used in the first experiment. Contaminated isolators were held at temperatures between 20 and 30 C during the 1-2-week interim between experiments.

Trial I. The inoculum used in trial I was initially prepared by using two groups of day-old poults (approximately 30 poults per group). One group was orally inoculated with the negative SS inoculum (1 ml/poult; see above). The other was administered the positive SS inoculum (1 ml/poult) by the same route. The two groups were housed and reared in separate containment isolators. On the fourth day post inoculation (PI), poults were euthanatized and their IECs were isolated and purified as described above. The final IECs concentration was adjusted to 10^{10} cells/ml in TPB.

The experimental design consisted of four groups of poults having 12 poults per group. One group (negative control group) was orally inoculated with TPB. The second group (positive control group) was administered the positive SS inoculum in the same manner. The third and fourth groups were orally administered the IECs isolated from negative SS- and positive-SS inoculated poults respectively. Each poult received 1 ml of inoculum. All groups were housed and reared in containment isolators for 10 days. The parameters of evaluation included body weights, specific intestinal maltase activity, clinical signs, and gross necropsy lesions.

Trial II. The inoculum used in trial II was initially prepared by using a group of day-old poults (approximately 30 poults) that were orally inoculated with the positive SS inoculum (1 ml/poult). The poults were housed and reared in containment isolators. On the fourth day PI, poults were euthanatized and their IECs were isolated and purified as described above. The IECs were lysed, filtered through 0.2-, 0.1-, and 0.02-μm filters, and centrifuged at 200,000 x *g* for 4 hr as described above.

The experimental design consisted of five groups of poults having 12 poults per group. One group (negative control group) was orally inoculated with TPB. The

second group (positive control group) was administered the positive SS inoculum in the same manner. Poults in groups 3, 4 and 5 were orally administered the IECs lysate filtrate (1 ml/poult) after it was passed through 0.2-, 0.1-, and 0.02-μm pore size filters respectively. All groups were housed and reared in containment isolators for 10 days. The parameters of evaluation included body weights, specific intestinal maltase activity, clinical signs, and gross necropsy lesions.

Trial III. The filtrate, from lysed IECs filtered through 0.1-m filters, was subjected to density gradient ultracentrifugation as described above (CsCl, sucrose and Accudenz®). The resulting fractions collected from each centrifugation media were pooled, dialyzed, and used for inoculation.

The experimental design consisted of five groups of poults having 12 poults per group. One group was (negative control group) orally inoculated with TPB. The second group (positive control group) was administered the positive SS inoculum in the same manner. Poults in groups 3, 4 and 5 were orally administered the pooled fractions from the sucrose, CsCl and Accudenz® centrifugation media, respectively. All groups were housed and reared in containment isolators for 10 days. The parameters of evaluation included body weights, specific intestinal maltase activity, clinical signs and gross necropsy lesions.

The IECs lysate prepared from both negative SS- and positive-SS inoculated poults and the fractions recovered from CsCl, sucrose and Accudenz® ultracentrifugation media were evaluated by EM.

Trial IV. The inoculum used in trial IV was prepared by using a group of day-old poults (approximately 30 poults). They were orally inoculated with the positive SS inoculum (1 ml/poult). The poults were housed and reared in containment isolators. On the fourth day PI, poults were euthanatized and their IECs were isolated and purified as described above. The IECs were lysed, filtered through 0.1-μm filter, and centrifuged at 200,000 x *g* for 4 hr. The pellet was subjected to density gradient

centrifugation using Accudenz® as described above. The resulting fractions (labeled 1 to 4 from top to the bottom of gradient) were dialyzed, pelleted, and used as an inoculum.

The experimental design consisted of six groups of poults having 12 poults per group. One group (negative control group) was orally inoculated with TPB. The second group (positive control group) was administered the positive SS inoculum in the same manner. Poults in groups 3, 4, 5 and 6 received inocula prepared from Accudenz® gradient fractions 1, 2, 3, and 4, respectively. All groups were housed and reared in containment isolators for 10 days. The parameters of evaluation included body weights, specific intestinal maltase activity, clinical signs, and gross necropsy lesions.

Trial V. Intestinal epithelial cell lysate filtrate (0.1 μm) was pelleted and subjected to density gradient ultracentrifugation using Accudenz® and CsCl. The fractions recovered were examined by EM and also orally inoculated to day-old poults. Five days PI, IECs were isolated and purified. The cells were lysed, filtered (0.1 μm), and centrifuged on Accudenz® gradient. Fractions were collected and examined by EM.

Trial VI. Primary culture of embryo IECs were prepared as previously described (2) with slight modifications. Briefly, the primary IECs were grown on homologous intestinal fibroblast feeder layers obtained from 14-to-16-day-old turkey embryos. The epithelial cells were isolated from day-old poults using type I collagenase (0.1%), and dispase (5 mg/ml) in supplemented media (MEM). The complete co-culture growth medium was supplemented with 20 units of preservative free heparin per 100 ml of medium.

The IEC lysates from negative and positive SS cells were filtered through 0.2-μm filters and activated with trypsin (trypsin type IX; 10 μg/ml) at 37 C for 1 h. Following trypsin activation, the trypsin activity was neutralized by the addition of trypsin inhibitor (type I-S) following the manufacturer's instructions. The cultured

cells were washed three times with serum-free supplemented MEM and were incubated with the activated virus suspension for 2 h at 37 C in 5% CO_2. The cells were washed again with MEM and low serum (1% each of FBS and chicken serum; pH 6.9) supplemented MEM was added. The infected cells were incubated for one week.

Following the fifth passage, the cells were dislodged from the flask by scrapping and were lysed as described above. The lysed suspension was centrifuged at 5000 x *g* for 30 min at 4 C. The supernatant was centrifuged at 200,000 x *g* for 4 hr at 4 C and the pellet was subjected to density gradient ultracentrifugation (using Accudenz®). The resulting visible bands were collected, dialyzed, pelleted by ultracentrifugation, and examined by EM (see above). These preparations were also used as inoculum.

The inoculum used in trial VI was prepared from primary IEC culture passaged SS inoculum as described above. The experimental design consisted of three groups of poults having 12 poults per group. Poults in group 1 received a preparation prepared from negative SS inoculated (cell culture-passaged) cells. Group 2 was orally administered the SS inoculum (see trial I) and served as positive controls. Group 3 was orally administered the preparations from cultured primary epithelial cells infected with SS as described above. All groups were housed and reared in containment isolators for 10 days. The parameters of evaluation included body weights, specific intestinal maltase activity, clinical signs, and necropsy lesions.

Statistical analyses. Body weight and specific intestinal maltase activity data were analyzed by the analysis of variance using crunch® software PC version 4 (Crunch Software Co., Oakland, CA). Differences between means for infected and corresponding controls were compared by the Student's t-test with a level of significance of $P \leq 0.01$ unless stated otherwise. For the trials involving multiple

groups, Tukey's test for multiple comparison was used with a level of significance of $P \leq 0.01$ unless stated otherwise. Only the groups within an experiment were compared.

RESULTS

Trial I. Poults inoculated with positive SS IECs had body weights and intestinal maltase activity significantly ($P \leq 0.001$) less than those of corresponding negative control poults at 10 days PI but were statistically identical to poults inoculated with positive SS (Fig. 1, 2). The poults inoculated with negative SS IECs did not develop SS, and the measured parameters were not different from that of negative control birds. The poults inoculated with positive SS IECs had lesions (watery gut contents, pale thin intestinal wall, loss of tonicity and cecal dilatation with brownish yellow frothy contents) very similar to positive SS-inoculated poults. The lesions were absent in both negative control and negative SS IEC-inoculated poults. Nearly identical findings were observed in the subsequent experiments when poults were infected via environmental exposure (Fig. 1, 2). The same results were obtained in a replicate for this trial (data not shown).

Trial II. The body weights and intestinal maltase activity of poults inoculated with 0.2 and 0.1 μm intestinal cell lysate filtrate were less ($P \leq 0.01$) than those of negative controls at 10 days PI (Fig. 3, 4). The weights, and maltase activity of poults in these groups were higher ($P \leq 0.01$) than those of positive SS-inoculated poults (Fig. 3, 4). The poults which received 0.2-, and 0.1-μm cell lysate filtrates had the intestinal lesions that were less severe when compared with the poults that received the SS inoculum. There was no dilatation of the ceca. The intestinal wall was thin with watery intestinal contents. Those poults inoculated with the 0.02-μm filtrate did not develop SS on the basis of weight gain, maltase activity (Figs. 3, 4),

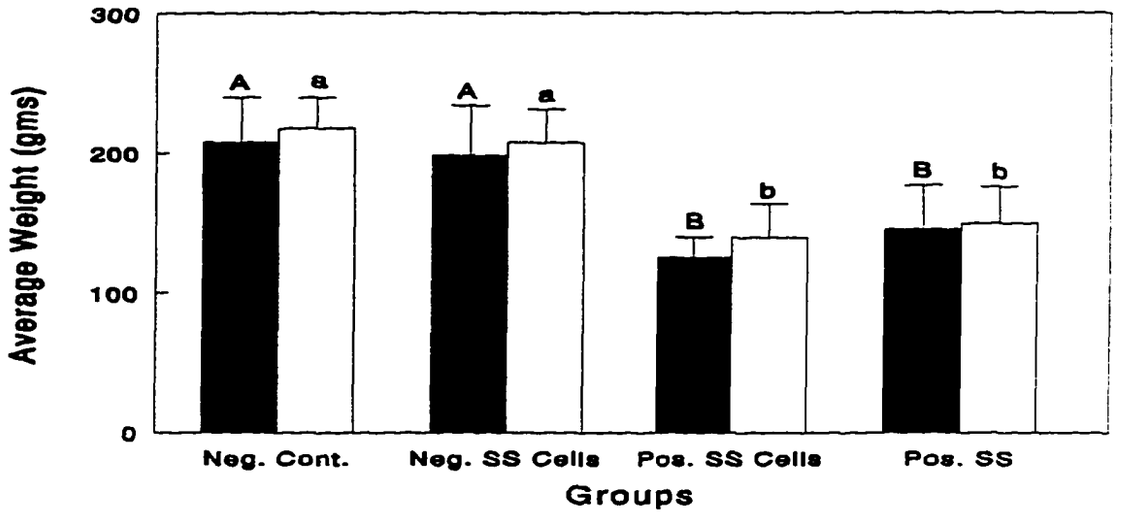

Fig. 1. Average body weight (gms) of poults (10 days PI) inoculated with TPB (Neg. Cont.), positive SS inoculum (Pos. SS) and purified IECs from negative SS (Neg. SS Cells) and positive SS inoculated (Pos. SS Cells) poults by oral route (■) and environmental exposure (□). Bars with different superscripts differ significantly at $P \leq 0.001$. Letters with same case represent statistical analyses within an experiment.

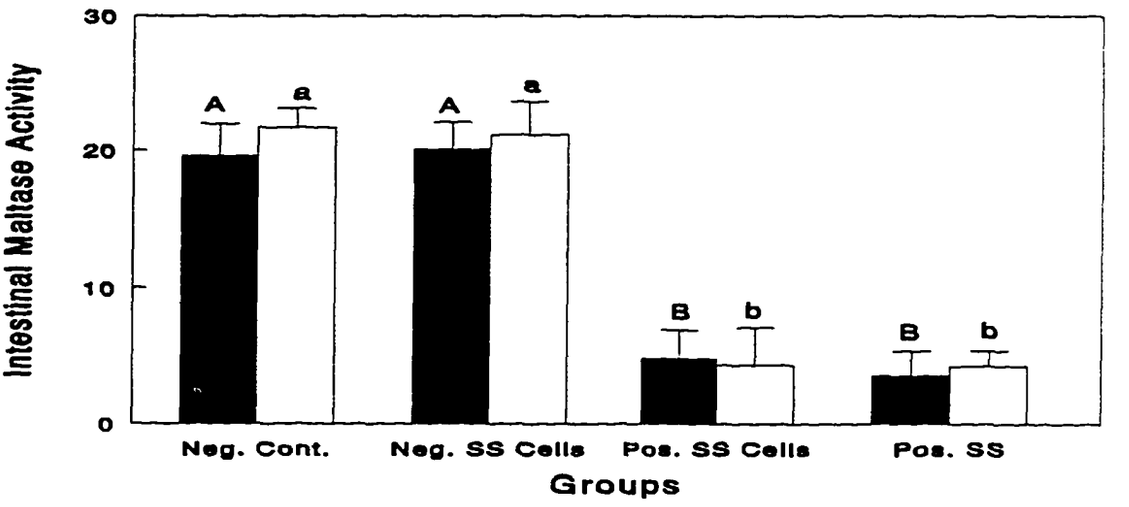

Fig. 2. Average specific intestinal maltase activity of poults (10 days PI) inoculated with TPB (Neg. Cont.), positive SS inoculum (Pos. SS) and purified IECs from negative SS (Neg. SS Cells) and positive SS inoculated (Pos. SS Cells) poults by oral route (■) and environmental exposure (□). Bars with different superscripts differ significantly at $P \leq 0.001$. Letters with same case represent statistical analyses within an experiment.

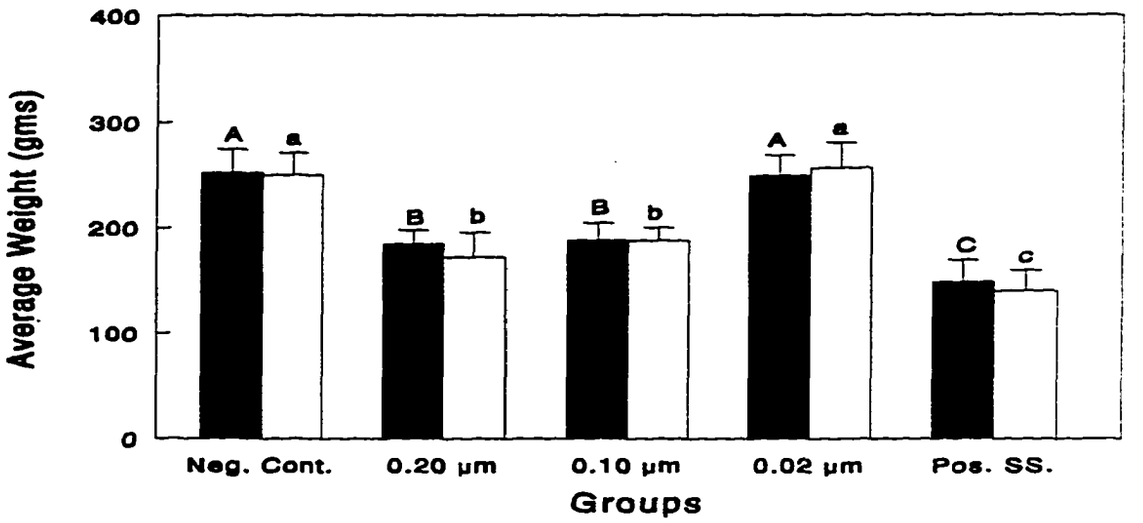

Fig. 3. Average body weight (gms) of poults (10 days PI) inoculated with TPB (Neg. Cont.), positive SS inoculum (Pos. SS) and purified IECs lysate after filtration through 0.2 (0.2 µm), 0.1 (0.1 µm) and 0.02 (0.02 µm) µm filters by oral route (■) and environmental exposure (□). Bars with different superscripts differ significantly at $P \leq 0.001$. Letters with same case represent statistical analyses within an experiment.

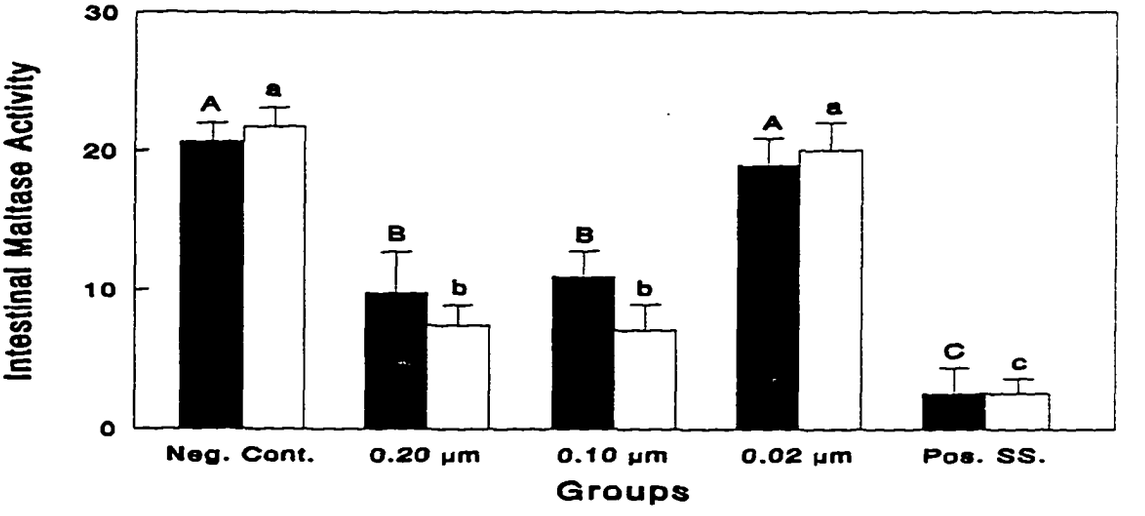

Fig. 4. Average specific intestinal maltase activity of poults (10 days PI) inoculated with TPB (Neg. Cont.), positive SS inoculum (Pos. SS) and purified IECs lysate after filtration through 0.2 (0.2 µm), 0.1 (0.1 µm) and 0.02 (0.02 µm) µm filters by oral route (■) and environmental exposure (□). Bars with different superscripts differ significantly at $P \leq 0.001$. Letters with same case represent statistical analyses within an experiment.

and gross intestinal lesions. Nearly identical findings were observed in the subsequent experiments when poults were infected via environmental exposure (Figs. 3, 4).

Trial III. The fractions collected and pooled following centrifugation on sucrose, CsCl and Accudenz® caused reduction in weight gain, and intestinal maltase activity. Among different density gradient media, Accudenz® was statistically the same as the positive SS inoculum (Figs. 5, 6). The intestinal gross lesions were similar to poults inoculated with the 0.1- or 0.2-µm filtrate (see trial II above). Nearly identical findings were observed in the subsequent experiments when poults were infected via environmental exposure (Figs. 5, 6).

Electron microscopic examination of IEC lysate from SS-positive birds revealed pleomorphic, membraned particles which ranged in size from 60 to 95 nm (Fig. 7). All the particles exhibited a membrane with relatively small, uniform projections. The interior parts of the particles, presumed to be the nucleocapsid, displayed varying types of morphology, including kidney-bean, dumb-bell, rod, and horse shoe shapes. Similar kinds of particles were observed in fractions from Accudenz® and sucrose gradient preparations. The particles were greater in number in fraction 2 from the Accudenz® preparation. The particles were observed in other fractions also, but were very low in number. The fraction, recovered following CsCl gradient centrifugation, had very few membraned particles.

Trial IV. There were four visible bands following ultracentrifugation of filtered (0.1 µm) IEC lysate on Accudenz®. The bands were quite diffuse (Fig. 8). The average densities of bands 1, 2, 3, and 4 were 1.089, 1.14096, 1.167 and 1.196 g/ml. The results of weight gain and maltase activity of poults inoculated with different fractions from Accudenz® gradient are shown in Figs. 9, 10. All the fractions produced SS but the decrease in weight gain and maltase activity was more pronounced, although statistically non significant, in poults inoculated with fraction 2

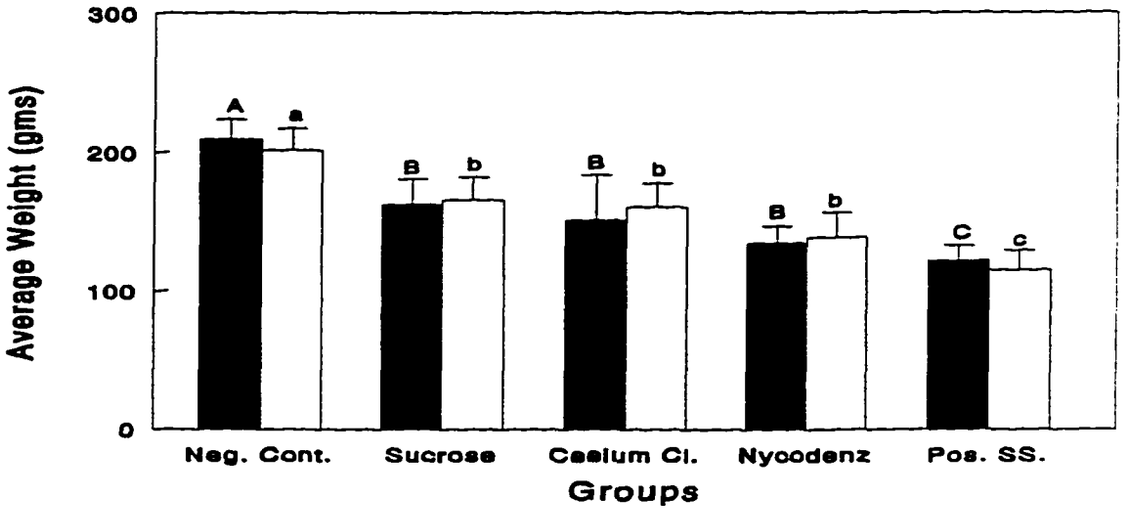

Fig. 5. Average body weight (gms) of poults (10 days PI) inoculated with TPB (Neg. Cont.), positive SS inoculum (Pos. SS) and fractions following ultracentrifugation from sucrose (sucrose), CsCl (CsCl) and Accudenz® (accudenz) density gradient media by oral route (■) and environmental exposure (□). Bars with different superscripts differ significantly at $P \leq 0.01$. Letters with same case represent statistical analyses within an experiment.

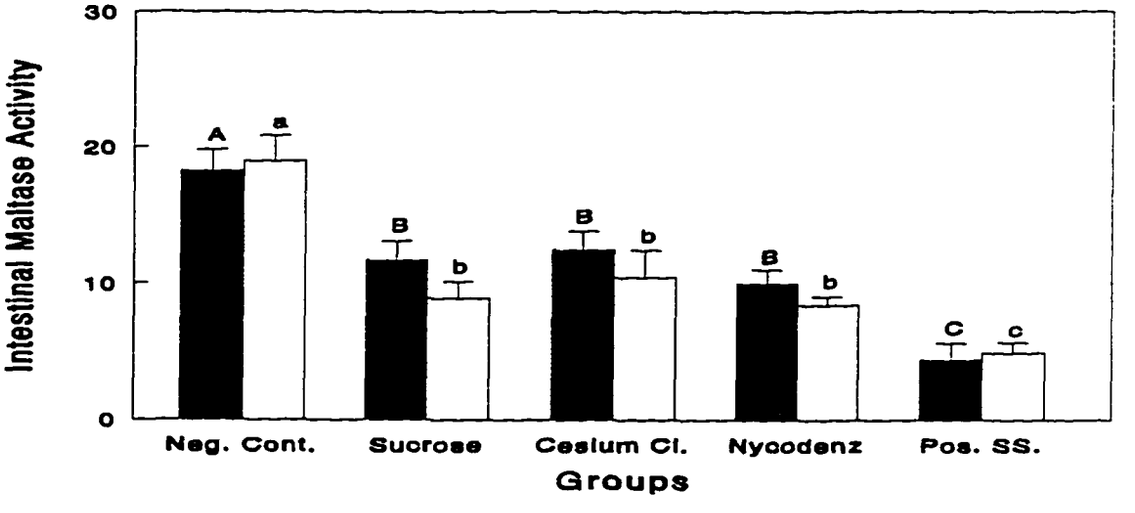

Fig. 6. Average specific intestinal maltase activity of poults (10 days PI) inoculated with TPB (Neg. Cont.), positive SS inoculum (Pos. SS) and fractions following ultracentrifugation from sucrose (sucrose), CsCl (CsCl) and Accudenz® (accudenz) density gradient media by oral route (■) and environmental exposure (□). Bars with different superscripts differ significantly at $P \leq 0.01$. Letters with same case represent statistical analyses within an experiment.

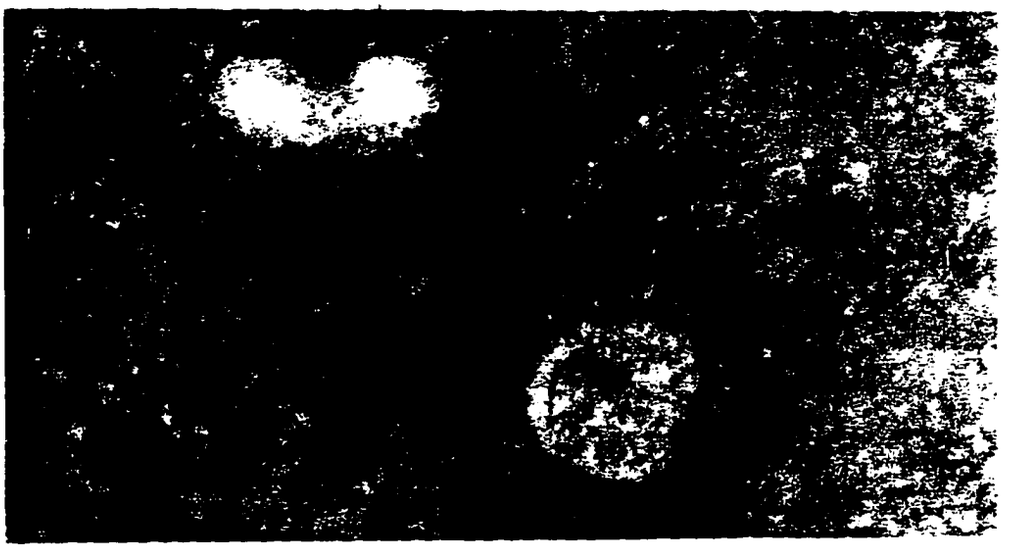

Fig. 7. Electron micrograph of negatively stained stunting syndrome agent (SSA) particles. The bar represent 100 nm.

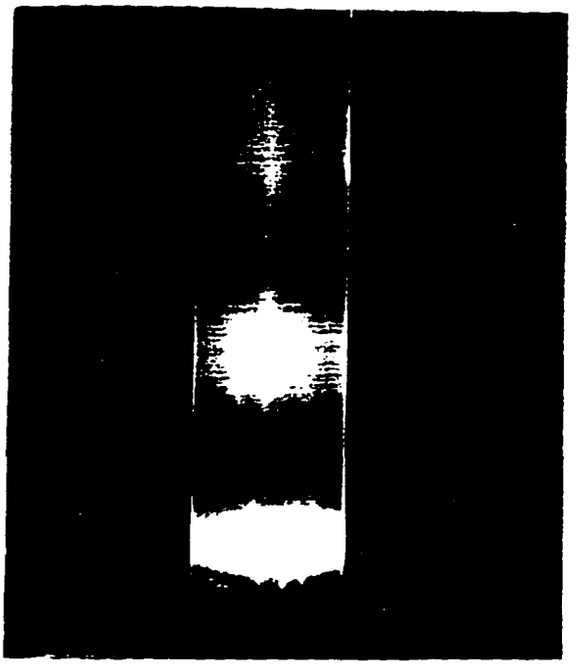

Fig. 8. Photograph of Accudenz® gradient showing different bands following ultracentrifugation of pellet from IEC lysate filtrate (0.1-µm filter).

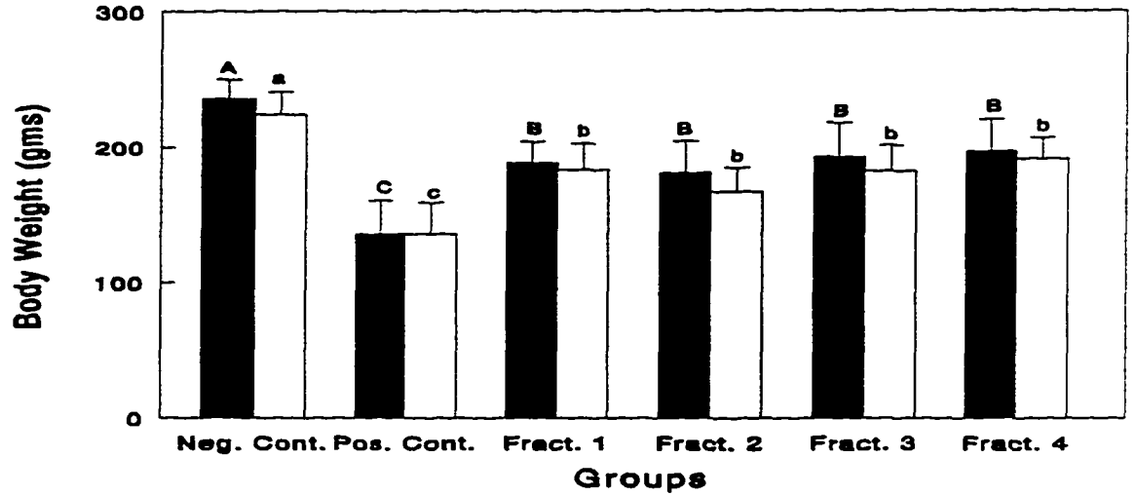

Fig. 9. Average body weight (gms) of poults (10 days PI) inoculated with TPB (Neg. Cont.), positive SS inoculum (Pos. SS) and fractions 1 through 4 (fract. 1, fract. 2, fract. 3 and fract. 4) following ultracentrifugation of pellet from IEC lysate filtrate (0.1-µm filter) on Accudenz® by oral route (■) and environmental exposure (□). Bars with different superscripts differ significantly at $P \leq 0.01$. Letters with same case represent statistical analyses within an experiment.

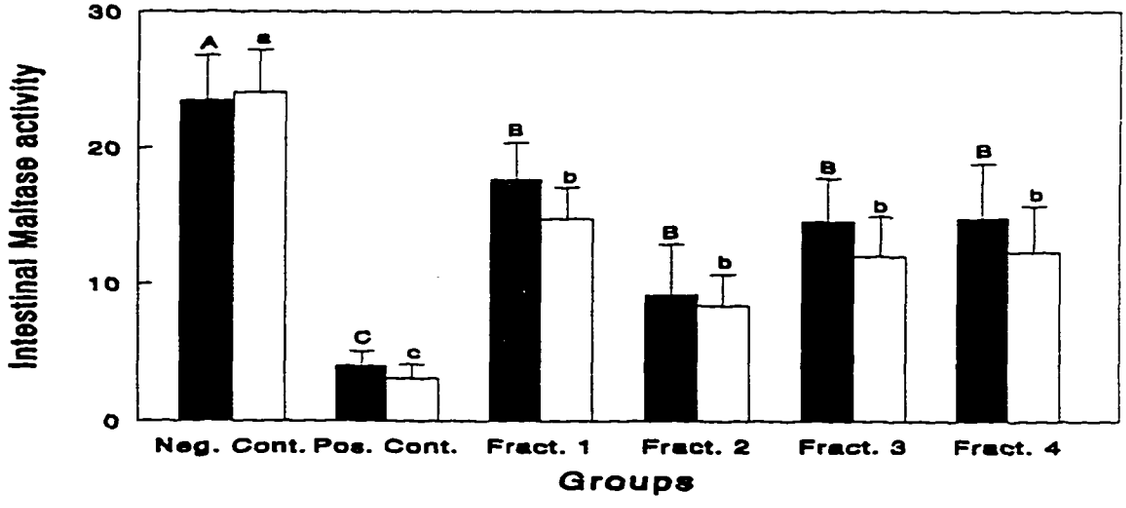

Fig. 10. Average specific intestinal maltase activity of poults (10 days PI) inoculated with TPB (Neg. Cont.), positive SS inoculum (Pos. SS) and fractions 1 through 4 (fract. 1, fract. 2, fract. 3 and fract. 4) following ultracentrifugation of pellet from IEC lysate filtrate (0.1 µm filter) on Accudenz® through oral route (■) and environmental exposure (□). Bars with different superscripts differ significantly at $P \leq 0.01$. Letters with same case represent statistical analyses within an experiment.

(Figs. 9, 10). The necropsy lesions included intestines that were pale, thin and contained watery contents. The lesions were more pronounced on days 4 and 5 PI. Nearly identical findings were observed in the subsequent experiments when poults were infected via environmental exposure (Figs. 9, 10).

Trial V. Poults inoculated with CsCl (one band; density 1.2858-1.298 g/cm^3) and Accudenz® (band #2) fractions in which particles were identified by EM developed clinical signs and lesions. The IECs isolated from inoculated poults (negative SS control, CsCl and Accudenz®) were lysed, filtered and subjected to ultracentrifugation on CsCl and Accudenz® media. Electron microscopic examination of fractions recovered from both gradients following ultracentrifugation revealed particles that appeared identical to those observed in the original inocula. These particles were not found in the cell lysate preparations from negative SS inoculated poults. These particles were also observed when the studies were repeated through environmental exposure.

Trial VI. Primary cultures of turkey IECs were used in an attempt to grow the Stunting Syndrome Agent (SSA) *in vitro*. No cytopathic effects were observed in SSA infected cells. After the fifth passage, the cell lysate was infectious for day-old poults. There was reduction in body weight and maltase activity (Figs. 11, 12). The necropsy lesions were similar to what were seen in poults inoculated with 0.1- or 0.2-μm filtrate (see trial II results above). Nearly identical findings were observed in the subsequent experiments when poults were infected via environmental exposure (Figs. 11, 12). The electron microscopic examination of cell lysate (after fifth passage from primary culture) revealed viral particles similar to those observed in the original inoculum from cell lysate filtrate.

DISCUSSION

Previous attempts in our laboratory to demonstrate, identify or isolate the SSA using conventional techniques such as EM, immune EM, egg-embryo

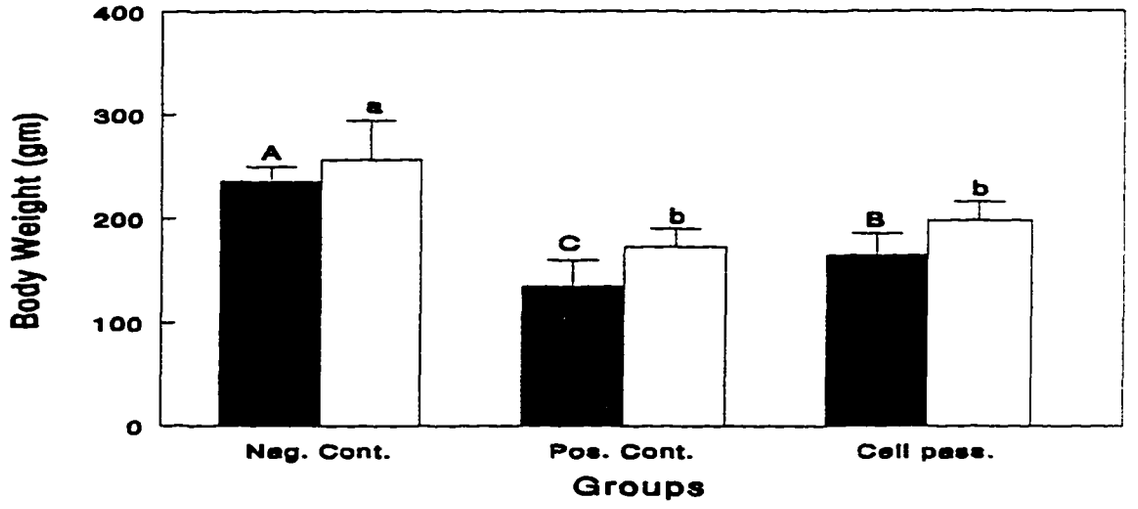

Fig. 11. Average body weight (gms) of poults (10 days PI) inoculated with positive SS inoculum (Pos. SS) and cell culture passaged (after fifth passage in primary IEC culture of turkeys) inoculum prepared from positive SS (cell pass.) and negative SS (Neg. Cont.) infected primary cells by oral route (■) and environmental exposure (□). Bars with different superscripts differ significantly at $P \leq 0.001$. Letters with same case represent statistical analyses within an experiment.

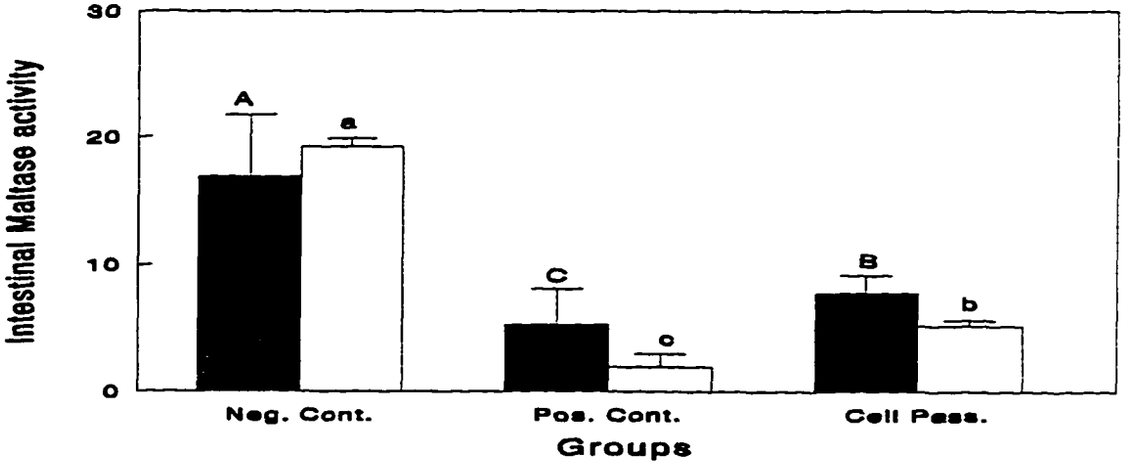

Fig. 12. Average specific intestinal maltase activity of poults (10 days PI) inoculated with positive SS inoculum (Pos. SS) and cell culture passaged (after fifth passage in primary IEC culture of turkeys) inoculum prepared from positive SS (cell pass.) and negative SS (Neg. Cont.) infected primary cells by oral route (■) and environmental exposure (□). Bars with different superscripts differ significantly at $P \leq 0.001$. Letters with same case represent statistical analyses within an experiment.

propagation, and cell culture techniques were unsuccessful (unpubl. data). Reasons that the conventional techniques were not successful in identifying the SSA may include dilution of virus in the feces/intestine, the nature of virus-cell association, the quantity and periodicity of viral shedding during the course of infection, unfamiliar morphology, poor seroconvertion (for immune EM), freezing and thawing of the intestinal samples, and improper sample storage. In an attempt to propagate the SSA, our laboratory developed a method for culturing IECs *in vitro* (2). We hypothesized that the SSA were associated with IECs. In order to prove this hypothesis, we exploited the methods used in the primary IEC culture technique (2), whereby IECs were isolated and purified from SS infected poults and subsequently used as an inoculum. It was clearly demonstrated in the first trial that SS was induced in susceptible poults by orally inoculating them with purified IECs from the intestines of SS infected poults but not from normal (negative SS inoculated) poults (Figs. 1, 2) thereby proving our hypothesis. The technique of using purified IECs from SS-infected poults proved to be of key importance in the isolation and purification of the SSA. Advantages for using purified IECs included being free from feed ingredients, digestive products, toxins, and microfloral organisms that may have been present in the intestine.

After we established that purified IECs transmitted SS, we resumed efforts to isolate and identify the etiologic agent. Attempts to grow bacteria (blood agar, McConkey's agar, Saboraud's dextrose agar, and Brucella agar [supplemented with 10% sheep blood, 20 µg/ml cefoperazone, 10 µg/ml vancomycin, 2 µg/ml amphotericin B and 1 % isovitalex; Becton Dickinson Labware, Bedford, MA) and mycoplasma (pleuro-pneumonia-like organisms agar) from lysed IECs were unsuccessful under aerobic, anaerobic and microaerophilic conditions (data not shown). These results strengthened the hypothesis that the SSA was a virus. To prove this hypothesis, we used conventional filtration techniques. IECs from SS

infected poults were lysed and filtered through 0.1-, and 0.2-μm filters. It was found that these filtrates caused SS (Figs. 3, 4). However, the severity of disease caused by the filtrates was less than that produced by SS inoculum. This decrease in the severity of SS was attributed to other factors associated with the SS inocula such as bacteria, or other agents, that may have been removed by filtration. It has been reported that bacteria contribute to the severity of the SS, but are not the primary cause (32). Bacteria-free filtrates (0.2 and 0.45 μm) of SS inoculum preparations produced the disease in susceptible poults (32). In the present study, there were no statistical differences in the weight gains and maltase activities of poults inoculated with either the 0.2-μm or 0.1-μm filtrate. The 0.02 μm filtrate did not produce any SS. On the basis of these results, the SSA ranged in size from 20 to 200 nm. This size range supports a viral etiology.

Nearly identical results were obtained in trials in which inoculation procedures were either by oral administration or by placement of poults in previously contaminated isolators (environmental exposure). The reason for including the environmental exposure route was to emulate natural exposure. Additionally, dose dependent responses are a concern when evaluating an infectious agent. Because there are no current methods to quantitate the SSA, environmental exposure was chosen to address this problem. The observation that SS developed in all the experiments following either oral administration or environmental exposure decreases the likelihood that SS is a disease caused by experimental conditions (i.e., dose) rather than a specific etiologic agent.

The SSA was purified using different ultracentrifugation density gradient media. Sucrose and CsCl are commonly used for the purification of non enveloped and enveloped viruses, respectively. Accudenz®, formerly known as Nycodenz® , is a non-ionic gradient medium that has been used most commonly for the separation and purification of subcellular particles (10, 27) and has not been widely used as a

medium for purifying viruses. In this study, Accudenz® was superior to sucrose and CsCl in retaining the infectivity of SSA and thus was selected for further use. Accudenz® medium is less viscous than sucrose at higher concentrations, and bands produced following ultracentrifugation are less diffuse than those produced with sucrose. Even though Accudenz® was superior to sucrose, pure SSA was very difficult to obtain because of contamination with cellular debris, membranes or other components with similar density. When the SSA particles were separated on CsCl and were observed by EM, most of the particles had lost their surface projections. This may be the reason for reduced infectivity that was observed in trial III. Although the SSA was isolated and identified using lysed IECs and ultracentrifugation methods, this method is not amenable for propagation of large quantities of SSA that may be desirable when developing diagnostic assays, antigens, etc. The *in vivo* method of harvesting IECs and *in vitro* method of culturing IECs may not be suitable for large scale production of SSA antigen because of the technical and economical limitations involved. Additionally, SSA purified by this procedure are contaminated with cellular debris which may interfere with its intended use. *In vitro* methods for the propagation of large quantities of SSA may prove useful for future studies.

Electron microscopic examination of the IEC lysate and the fractions recovered following density gradient ultracentrifugation revealed membraned particles of various shapes and sizes (Fig. 7). A definitive identification of the SSA could not be made based on size and morphology. A number of membraned viruses including coronaviruses (22), toroviruses (14), orthomyxoviruses (8), paramyxoviruses (1) have the capability of replicating in the intestinal tract. Some of these viruses produce enteric disease in various animal species. On the basis of EM, the SSA was tentatively identified as a coronavirus, the etiologic agent of turkey bluecomb disease (22). However, preliminary data (hemagglutination and

serological analysis) in our laboratory suggest that the SSA is definitely different from the enteric coronavirus (bluecomb disease agent). Another distinguishing characteristic between SSA and bluecomb disease agent is the biologic characteristic of age susceptibility. Although the bluecomb disease agent reportedly causes disease in turkeys of any age (22), the SSA has a definite age susceptibility. Day-old poults are most susceptible to SS but poults become increasingly refractory to the disease and by three weeks of age, poults are no longer susceptible (J. L. Sell, pers. comm.). Coronaviruses have been speculated to be involved in SS but their exact role is currently unknown. The morphology of SSA has some resemblance to toroviruses having disc-, kidney-, or rod-shape morphology of the nucleocapsid (14). The toroviruses and torovirus like particles have been observed in feces from diarrheic cattle (34), humans (5), dogs (9), cats (20), swine (30), and other animals and have been reported to cause diarrhea in experimentally infected calves (35). Serologic antibodies to toroviruses have also been detected in sheep, goat, horses, laboratory rabbits and mice (33). Although toroviruses have not been reported in turkeys, the potential of their occurrence cannot be discounted. Orthomyxoviruses (8) and paramyxoviruses (1) are wide spread in avian species, including turkeys, but their potential involvement in SS has not been reported. Fringed membranous particles have been observed by EM in both healthy and diseased (poult enteritis) poults (11) and might be confused with membraned viruses but their cause and effect relationship has not been established. Further studies to properly classify the SSA are needed.

To establish the cause and effect (fulfillment of Koch's postulates) relationship for the SSA, trials V and VI were performed. Koch's postulates state (6) that 1) the agent must be present in every case of the disease 2) the agent must be isolated from the host and grown *in vitro* 3) the disease must be reproduced when a pure culture of the agent is inoculated into a healthy susceptible host, and 4) the

same agent must be recovered once again from the experimentally infected host. In trial V, poults inoculated with purified preparations of SSA developed SS, and the agent was recovered from the affected poults thus fulfilling Koch's postulates 3 and 4. In the trial VI, the SSA was propagated in primary cell culture of turkey intestinal epithelium. Following the fifth passage, the SSA inoculated cell culture preparations produced SS in day-old turkey poults, fulfilling Koch's postulates 2, 3, and 4. Additionally the propagation of the SSA in cell culture decreased the likelihood of the SSA inoculum harboring adventitious agents. This was corroborated by electron microscopy evaluation of the SSA inoculum prepared from the cell culture preparations in which no viral particles or other agents other than SSA were observed. The technique of primary intestinal epithelial cell culture proved useful in the isolation of SSA. This technique may also be useful for the isolation and identification of other fastidious enteric agents.

In summary, the technique of using purified IECs from SS infected poults was of key importance in the isolation and purification of SSA. This technique may also have applications in the isolation and identification of other fastidious enteric pathogens. The employment of Accudenz®, which has self-generating gradient properties, was useful during the isolation and purification of SSA. Accudenz® was more advantageous than sucrose in this study and may be useful in other applications. The primary intestinal epithelial cell culture technique was used for the initial isolation of the SSA. Although this technique may not be economically feasible for routine use, it may be useful for the isolation of other enteric pathogens. The results of this study provide evidence that a previously unidentified but yet to be classified viral agent has been isolated and identified from IECs of infected poults and is the etiologic agent of SS. Further studies to properly classify the SSA are needed.

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CHAPTER 5. THE *IN VITRO* PROPAGATION OF STUNTING SYNDROME AGENT

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SUMMARY. Stunting Syndrome (SS) is an enteric disease of turkey poults resulting in high morbidity and is manifested by reduced growth, impaired feed efficiency and diarrhea. The etiologic agent of this disease has recently been reported as being a pleomorphic, membraned virus (stunting syndrome agent; SSA). The objective of this study was to develop a method for *in vitro* propagation of SSA. Primary cells, various continuous cell lines and embryonated eggs were evaluated. Turkey embryos that were inoculated via the amniotic cavity at 24-to-25-days of incubation were susceptible to SSA infection. The jejunal maltase activity of SSA inoculated turkey embryos was significantly ($P \leq 0.001$) lower than control embryos. D-xylose absorption was also altered in SSA infected turkey embryos. The extent of reduction of D-xylose absorption and maltase activity in the infected embryos were nearly identical to those observed when day-old poults were infected with SSA. The intestines from the infected turkey embryos were pale, thin walled and distended with fluid. Electron microscopic (EM) examination of the intestinal fluid and epithelial cell lysate of infected embryos revealed pleomorphic membraned SSA viral particles. It was found that SSA which had been serially passaged in turkey embryos retained its ability to induce SS in day-old poults. All the primary and continuous cells which were evaluated did not support the replication of SSA on the basis of cytopathic effects, EM and turkey embryo inoculation. The results of this study indicate that the SSA was successfully propagated in turkey embryos which exhibited alterations in embryo intestinal absorption and digestive enzyme activity similar to poults with SS. Successful propagation of SSA in turkey embryos should

prove beneficial for future studies involving characterization of SSA, prevention and control strategies, enteric disease modeling, etc.

Key words. Stunting syndrome; *in vitro* propagation; poult enteritis; turkey viral enteritis; intestinal maltase activity; D-xylose absorption; stunting syndrome agent.

Abbreviations: EDTA = ethylenediaminetetraacetic acid, FBS = fetal bovine serum, HEPES = (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), IECs = intestinal epithelial cells, SS = stunting syndrome, SSA = stunting syndrome agent, CPE = cytopathic effects, PI = post inoculation, CAM = chorioallantoic membrane, EM = electron microscopy, TPB = tryptose phosphate broth, .

INTRODUCTION

An enteric disease in young turkeys has been previously reported as stunting syndrome (SS [3, 16]). Typically, the onset of clinical disease occurs between 1 and 3 weeks of age. The most common manifestations of SS include poor growth, decreased feed utilization, depression, diarrhea, and enteritis (13, 16). The gastrointestinal tract appears to be a primary target organ. Typical necropsy lesions include cecal dilatation with gaseous yellow to brown contents and pale, thin intestines with watery contents (16, 18). The etiologic agent of this disease has recently been reported as an unclassified pleomorphic, membraned virus referred to as stunting syndrome agent (SSA). The SSA produces enteric disease in day-old turkey poults causing reduction in weight gain, maldigestion, diarrhea and enteritis (2).

Studies on the pathogenesis, diagnosis and prevention of diseases caused by enteric viruses have often been hampered by the inability to isolate and/or propagate these viruses *in vitro*. Two commonly used *in vitro* methods for the isolation of viruses include tissue culture (primary, and continuous cells) and embryonated eggs. Although SSA has been successfully propagated in primary

intestinal epithelial cell culture (2), the economic and technical difficulties of this method limits its use on a regular and/or large scale basis.

The objectives of this study were to establish and evaluate an *in vitro* system for the propagation of SSA.

MATERIALS AND METHODS

Eggs and Poults. Fertile turkey eggs were procured from a commercial hatchery. Chicken eggs were procured from a SPF source (Hy-Vac Laboratory Eggs Co., Adel, IA). The eggs were incubated under standard conditions. Day-old commercial turkey poults were used and reared in plastic positive pressure containment isolators as previously described (2). Feed and water were provided *ad libitum*.

Preparation of SS inoculum. Two groups of day-old poults (approximately 30 poults per group) were used for inoculum preparation. One group was orally inoculated with a negative SS inoculum (1 ml/poult) prepared from the intestines of normal poults as described previously (2, 3). The other group of poults was administered a positive SS inoculum (1 ml/poult) by the same route. The positive SS inoculum was prepared from the intestinal tract of SS infected poults. The two groups were housed and reared in separate containment isolators. On the fourth day post inoculation (PI), poults were euthanatized and their intestinal epithelial cells (IECs) were isolated and purified as described previously (1). The cell concentration was adjusted to 10^{10} cells/ml and lysed using Parr® 4635 cell disruption bomb (Parr Instrument Co., Moline IL). The cell lysate was serially passed through filters of different pore size. Filtrates after passing through 0.2-µm pore size filters were used in subsequent studies.

Trypsin activation of SSA in cell lysate filtrate was carried out in the presence of type IX trypsin (10 µg/ml; Sigma Chemical Co., St Louis, MO) at 37 C for 1 hour. The mixture was cooled immediately on ice and left on ice until further used. Tryptose phosphate broth (TPB) was used as negative control inoculum.

Measurement of Intestinal Maltase activity. Embryo intestinal samples (entire jejunal length) were frozen immediately on crushed dry ice. Poults were euthanized and the jejuna were removed. The jejuna were flushed with ice chilled PBS and the intestinal tissue was frozen immediately in liquid nitrogen. The jejunal tissues were prepared for further analysis as described previously (20) with slight modification. The jejunal tissue was homogenized in distilled water (1:10 for the embryos and 1:20 for the poults). The total protein was determined by the method of Lowry (12). Maltase activities were determined by the method of Dahlquist (4). The specific intestinal maltase activity was calculated as the enzyme activity based on percent protein.

Electron microscopy. Samples were diluted appropriately (1:2 to 1:8) with sterile distilled water and stained with an equal volume of phosphotungstic acid (PTA) solution (PTA 3%, sucrose 0.4%; pH 6.0) solution for 60 sec. The virus-PTA mixture was then applied to Formvar®, (Electron Microscopy Sciences, Fort Washington, PA) carbon-coated 300-mesh copper grids. The grids were observed under a Hitachi-500 electron microscope at 75 KV.

Density gradient centrifugation. Density gradient centrifugation using Accudenz® (5-(2,3-dihydroxypropylacetamido-2,4,6-tri-iodo-N,N'-bis (2,3 dihydroxypropyl)) isophthalamide; Accurate Chemical & Scientific Co., Westbury, NY) was performed to purify the virus. A discontinuous Accudenz® step gradients was made by successively layering 50%, 40%, 30%, 20%, and 10 % Accudenz® solutions (in TNEM buffer; 10 mM tris, 0.01 M NaCl, 2 mM $MgCl_2$ and 0.002 M EDTA; pH 7.0). The sample was applied on top of the gradient. The gradient was centrifuged at 200,000 x *g* for 16 hr at 4 C. Following centrifugation, visible bands were harvested and dialyzed against 5-6 changes of TNC (10 mM tris, 0.01 M NaCl, 20 mM $CaCl_2$; pH 7.0) buffer at 4 C. Each fraction was examined for SSA particles by EM.

Primary cell culture. Primary cell cultures of chicken and turkey embryo fibroblasts, kidney, liver and pancreas were evaluated for propagation of SSA. Chicken and turkey embryo fibroblasts and kidney cells were cultured following a described procedure (19) using Dulbecco's modified eagles medium (DMEM; Life Technologies Inc., Gaithersburg, MD) supplemented with 5% fetal bovine serum (FBS), 5% chicken serum, and 1% penicillin-streptomycin-fungizone (PSF; Bio-Whittaker Inc., Walkersville, MD) mixture. Cells from embryo liver were cultured following a described procedure (11) with slight modifications. The liver cells were isolated using 3 mg/ml collagenase (hepatocyte qualified; Life Technologies Inc.) in digestion buffer (3 mM KCl, 160 mM NaCl, 1 mM NaH_2PO_4. H_2O, 1.82 g/l glucose and 1 % ITS plus® ; Becton Dickenson Labware, Bedford, MA). The isolated cells were grown in William's medium E (Fisher Scientific, Pittsburgh, PA) supplemented with FBS (5%), chicken serum (5%), PSF mixture (1%) and ITS plus™ (1%). Pancreatic acini cells were prepared and cultured following a previously described method (8). All the growth media were supplemented with 2 mM L-glutamine prior to use. Cells were grown in 5% CO_2 at 37 C and were infected 48 to 72 hr after the monolayers reached confluency.

Continuous cells. Embryonic African green monkey kidney (MA-104), human ileocecal adenocarcinoma (HRT-18), human colon adenocarcinoma (Caco-2), buffalo green monkey kidney (BGM-70), African green monkey kidney (LLC MK2), Madin-Darby canine kidney (MDCK), African green monkey kidney (Vero), bovine turbinate (BT), normal rat liver (Clone 9), pig kidney (LLC PK_1), porcine fetal testis (ST), and quail turbinate (QT35) cells were used in an attempt for SSA propagation. The cells were grown in their respective recommended media. The cells were allowed to reach 90-100 % confluency and were infected at 48-72 h post confluency.

Embryo inoculation. Both chicken and turkey embryos were inoculated through different routes including allantoic cavity, chorioallantoic membranes (CAM), yolk sac and amniotic route. The time and route of inoculation are indicated in table 1. Both chicken and turkey embryos were injected via allantoic cavity, CAM, and yolk sac following a described procedure (21). Following injection, the eggs were incubated under standard incubation conditions. Injection via amniotic cavity was made following a described procedure (15) with modifications. A circular hole of 1.8 cm diameter was drilled through the egg shell over the air sac. The shell and shell membrane were reflected to one side. Before injection, 2-3 drops of sterile 15% ethyl alcohol (v/v in deionized water) were dropped onto the embryonic membranes to allow visualization of blood vessels and other embryonic structures. A 26-gauge needle was inserted just below the embryonic membranes avoiding any major blood vessels and/or other embryonic structures. The inoculum (0.2 ml) was injected into the amniotic cavity. The hole in the shell was sealed using a self adhesive clear pressure sensitive film (Becton Dickenson). The eggs were incubated in a hatcher under standard hatching conditions until removed for further studies.

D-Xylose absorption test in embryos and poults. The D-xylose absorption test was performed following previously described procedures (7, 9). Briefly, the embryos were partially removed from their shells so that there heads and necks were accessible. A 5% sterile solution of D-xylose (Sigma) was given orally at the rate of 10 ml/kg of total egg weight. Blood samples were collected via jugular venipuncture in heparinized capillary tubes. Following D-xylose administration, the embryos were placed back into the incubator except when removed for blood samples collection at 30, 60, 90 and 120 min for D-Xylose determination.

Poults were fasted for 5-6 h prior to testing. Blood was collected at 90 min following D-xylose administration in heparinized capillary tubes for D-xylose determination.

Table 1. Time table of inoculation of chicken and turkey embryos with SSA by different routes.

Embryo species	Inoculation routes	Inoculation days	Time of evaluation (DPI)[1]	Specimen(s) collected	Evaluation parameters
Chicken	CA[2]	9-to-11	7	allantoic fluid, CAM membranes, embryo intestines	lesions, EM
	CAM[3]	9-to-11	7	allantoic fluid, CAM membranes, embryo intestines	lesions, EM
	YS[4]	7-to-8	7 & 11	yolk, yolk sac, embryo intestines	lesions, EM
	AM[5]	17	4-5	embryo intestines, intestinal fluid	lesions, EM, maltase
Turkey	CA	11-to-13	7	allantoic fluid, CAM membranes, embryo intestines	lesions, EM
	CAM	11-to-13	7	allantoic fluid, CAM membranes, embryo intestines	lesions, EM
	YS	9-to-11	7 & 15	yolk, yolk sac, embryo intestines	lesions, EM,
	AM	23-24	3-4	embryo intestines, intestinal fluid	lesions, maltase, D-xylose abs.[6] EM

[1] Days post inoculation.
[2] Chorioallantoic cavity.
[3] Chorioallantoic membranes.
[4] Yolk sac.
[5] Amniotic cavity.
[6] D-xylose absorption test.

Experiment I. In experiment I, embryonated turkey and chicken eggs were inoculated by different routes as indicated in table 1. Turkey or chicken embryos were divided into four groups of 10-30 eggs per group for each inoculation route. One group served as uninoculated control while the second group was injected with 0.2 ml of TPB to serve as inoculated negative control group. The third and fourth groups were injected (0.2 ml/egg) with trypsin activated negative SS IECs (neg. SS) and trypsin activated positive SS IECs (pos. SS) lysate filtrate respectively. Following inoculation, eggs were incubated as described above and candled daily. The egg inoculations were repeated for a total of five passages. Following the fifth passage, IECs were isolated from half of the embryos. Subsequently, IECs were lysed, filtered (0.1 μm), and subjected to density gradient ultracentrifugation as described above. For embryos inoculated via the CAM and allantoic cavity, the CAM and amniotic membranes were harvested and homogenized with PBS (1:10 dilution). The homogenates were filtered and subjected to density gradient centrifugation. The intestinal, allantoic, and amniotic fluid was also harvested separately and subjected to density gradient ultracentrifugation. The fractions collected were examined by EM. The jejuna were collected from the remaining half of the embryos for maltase determination. Additionally some embryos inoculated via the amniotic route were evaluated for D-xylose absorption.

Experiment II. The experiment II was performed to determine the effect of trypsin activation on SSA. The cell culture lysate filtrate was trypsin activated as described above. The SSA with and without trypsin activation were serially (10 fold) diluted in TPB. Five embryos were injected (0.2 ml/embryo) for each dilution via the amniotic cavity. The control groups consisted of TPB (negative control), uninoculated control, trypsin (10 μg/ml type IX trypsin) and undiluted IECs lysate filtrate (positive control). The jejuna were collected after 72 hr of injection for maltase determination. The experiment was repeated.

Experiment III. The pathogenic effects of the embryonated egg passaged SSA were compared to an intestinal homogenate inoculum derived from SS infected poults. The experimental design consisted of 3 groups of poults with 12 poults per group. One group (negative control) was orally inoculated with 1 ml of TPB per poult. Poults in group two (positive control group) were orally inoculated (1 ml/poult) with SS inoculum (see above). The poults comprising the third group were orally administered (1 ml/poult) the filtered (0.1 µm) intestinal fluid harvested from the turkey embryos which had been inoculated via the amniotic route (following five passages). Poults in all the groups were housed and reared in separate containment isolators. Feed and sterile water were provided *ad libitum*. Parameters of evaluation included weight gain, EM, intestinal maltase activity and D-xylose absorption at 9 days PI. The experiment was repeated.

Experiment IV. In an attempt to propagate SSA in cell culture, cells (primary and continuous) were prepared in 25 cm^2 T-flasks (Corning Inc., Corning, NY). The monolayers (48-72 h post confluency) were washed three times with their respective serum-free media. The monolayer of each cell type was infected under five different set of conditions (treatments). In all cases, the inoculum source was IECs lysate filtrate (0.2 µm). The five different treatments were:-

1. One set of flasks was infected with trypsin activated virus suspension. The cell lysate filtrate (0.2 µm) was incubated with type IX trypsin (10 µg/ml) at 37 C for 1 hr. Following incubation, the trypsin activity was neutralized by the type I-S trypsin inhibitor (Sigma) following the manufacturer's instructions. Respective serum-free media and negative SS IECs lysate filtrate were subjected to the similar treatments to serve as corresponding negative controls. The activated virus suspension, negative control medium and negative SS IECs lysate filtrate (0.8 ml) was applied to the cell monolayers. The monolayers were incubated for 2 hr at 37 C in 5% CO_2.

The monolayers were washed and maintenance medium (respective serum-free media) with 1 µg/ml of type-IX trypsin was added.

2. The inocula used was not trypsin activated but trypsin was included in the maintenance medium (1 µg/ml). The remainder of the procedure was the same as described above.

3. The inocula used was not trypsin activated and pancreatin (Sigma; 100 µg/ml) was included in the maintenance medium.

4. Cells were infected with inocula (without trypsin activation) and their respective serum-free media, without trypsin/pancreatin supplementation, were used for cell maintenance.

5. Following a passage of cells in antibiotic-free media, the cells were inoculated with SSA (without trypsin activation) and antibiotic-free maintenance media was used.

Following inoculation, the cells were incubated at 37 C for 7 - 10 days in 5% CO_2. The cells were examined daily for cytopathic effects (CPE). At the end of incubation, the cells were frozen (-70 C) and thawed once and centrifuged (10,000 x *g* for 30 min at 4 C). The supernatant was used to re-infect cells. Five blind passages were performed with each cell type. Following the fifth passage, the cells were frozen, thawed and centrifuged (10,000 x *g* for 30 min at 4 C) before density gradient ultracentrifugation (as described above). The cell lysate was also injected (0.2 ml) into 24-to-25-day-old turkey embryos via the amniotic route as described above. The infection in the embryo was assessed on the basis of EM, intestinal lesions and embryo intestinal maltase activity.

Statistical analyses. Specific intestinal maltase activity, body weight and D-xylose absorption data were analyzed by the analysis of variance using Statistix® software for windows version 1 (Analytical Software, Tallahassee, FL). Differences between means for infected and corresponding controls were compared by the Student's t-

test with a level of significance of $P \leq 0.01$ unless stated otherwise. For experiments with multiple groups, Tukey's test for multiple comparison was used with a level of significance of $P \leq 0.01$ unless stated otherwise.

RESULTS

Experiment I. The inoculation of embryonated chicken eggs via the allantoic cavity, yolk sac, CAM, and amniotic cavity did not result in SSA replication. The results are presented in table 2. The intestinal maltase activity was normal in SSA infected chicken eggs inoculated through the amniotic cavity (Fig. 1). Inoculation of turkey embryos by the allantoic cavity, CAM, or yolk sac route failed to support SSA replication on the basis of the evaluated parameters (table 2). However, SSA replication was detected when turkey embryos were inoculated via the amniotic route. The intestines from the infected embryos were fragile, pale, thin-walled and filled with fluid (Fig. 2). The amount of fluid in the intestinal tract varied from 1.5 to 4.0 ml. The ceca were markedly dilated and filled with greenish watery fluid. The intestinal maltase activity was significantly ($P \leq 0.001$) reduced in embryos inoculated with SSA (Fig. 1). The results of D-xylose absorption are presented in fig. 3. The D-xylose absorption was reduced in embryos inoculated with SSA through the amniotic route.

Experiment II. The results of trypsin activation of SSA are shown in fig. 4. Trypsin activation resulted in 2-3 log increase in virus infectivity on the basis of lesions and intestinal maltase activity.

Experiment III. The body weight of poults inoculated with embryo passaged SSA (following fifth passage) were significantly lower ($P \leq 0.01$) than control poults at 9 days PI (Fig. 5A). Poults that received embryo passaged SSA had decreased intestinal maltase activity (Fig. 5B). Similarly, the D-xylose absorption was also significantly reduced when compared to control poults at 9 days PI (Fig. 5C). SSA

Table 2. The results of infection of chicken and turkey embryos inoculated with SSA by different routes.

Embryo species	Inoculation routes	Specimen(s) collected	Results			
			Lesions[1]	EM[2]	Maltase	D-xylose[3]
Chicken	CA[4]	allantoic fluid, CAM, embryo intestines	-	-	ND[5]	ND
	CAM[6]	allantoic fluid, CAM, embryo intestines	-	-	ND	ND
	YS[7]	yolk, yolk sac, embryo intestines	-	-	ND	ND
	AM[8]	embryo intestines, intestinal fluid	-	-	NE[9]	ND
Turkey	CA	allantoic fluid, CAM, embryo intestines	-	-	ND	ND
	CAM	allantoic fluid, CAM, embryo intestines	-	-	ND	ND
	YS	yolk, yolk sac, embryo intestines	-	-	ND	ND
	AM	embryo intestines, intestinal fluid	+	+	Decreased[10]	Decreased

[1]Gross intestinal lesions.
[2]Presence of SSA by Electron Microscopy.
[3]D-xylose absorption.
[4]Chorioallantoic cavity.
[5]Not done.
[6]Chorioallantoic membranes.
[7]Yolk sac.
[8]Amniotic cavity.
[9]No effect.
[10]Decreased compared to control ($P \leq 0.001$).

particles were detected in IECs lysate from poults inoculated with embryo passaged SSA.

Experiment IV. In experiment IV, different primary and continuous cultured cells were infected with SSA under different treatment conditions. Inoculation of these primary and continuous cells did not result in virus replication on the basis of CPE and EM. After 5 passages in cell cultures, inoculation of 24-to-25-day-old turkey embryos via the amniotic cavity did not result in virus replication on the basis of EM, intestinal lesions, or decreased intestinal maltase activity.

DISCUSSION

The etiologic agent of stunting syndrome (SS) has recently been reported (2) and termed stunting syndrome agent (SSA). Although this viral agent was isolated in primary cultures of intestinal epithelial cells (2), the technical and economic limitations of this cell culture system hampers its use for routine and/or large-scale virus propagation. To develop an alternative method for propagation of SSA was a

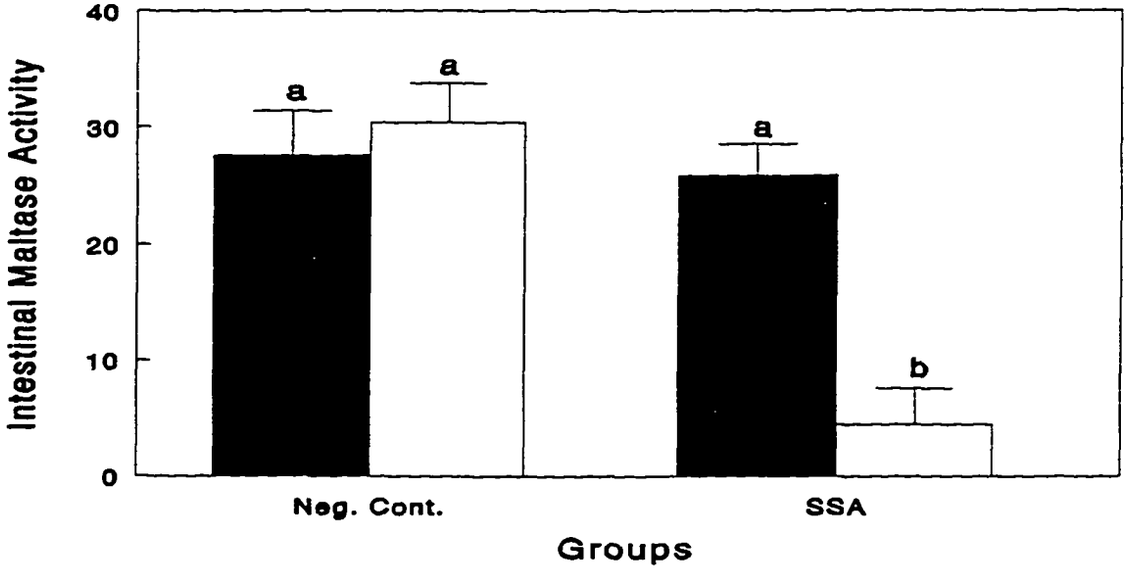


Fig. 1. Specific intestinal maltase activity of chicken (■) and turkey (□) embryos inoculated with TPB (Neg. Cont.) and stunting syndrome agent (SSA). Bars with different superscript differ at $P \leq 0.0001$.

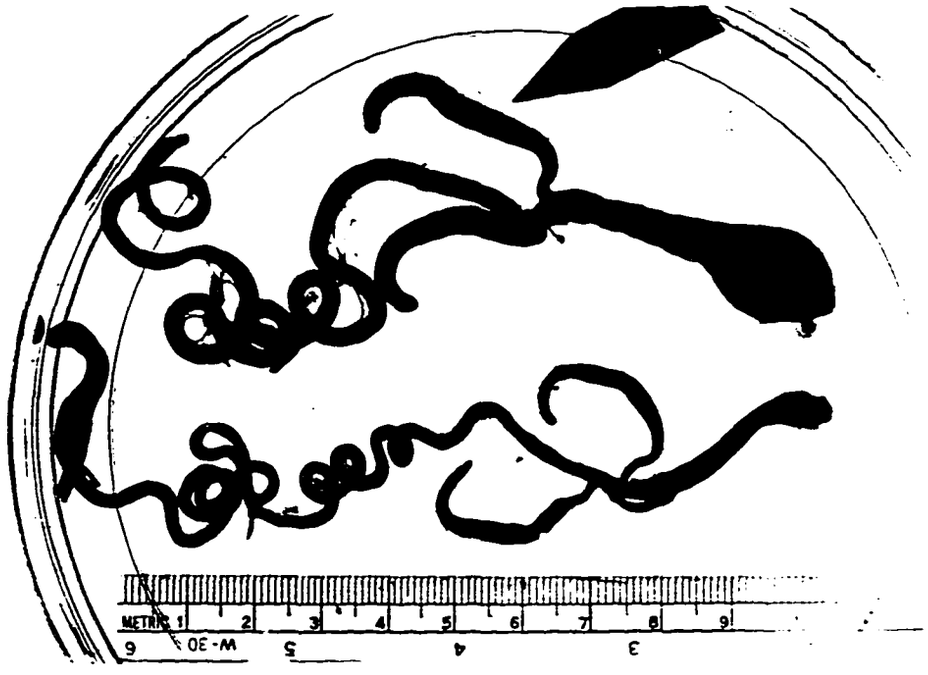


Fig. 2. Intestines from 24-to-25-day-old turkey embryos inoculated with stunting syndrome agent (top with arrow) and tryptose phosphate broth (bottom) at 72 h post inoculation.

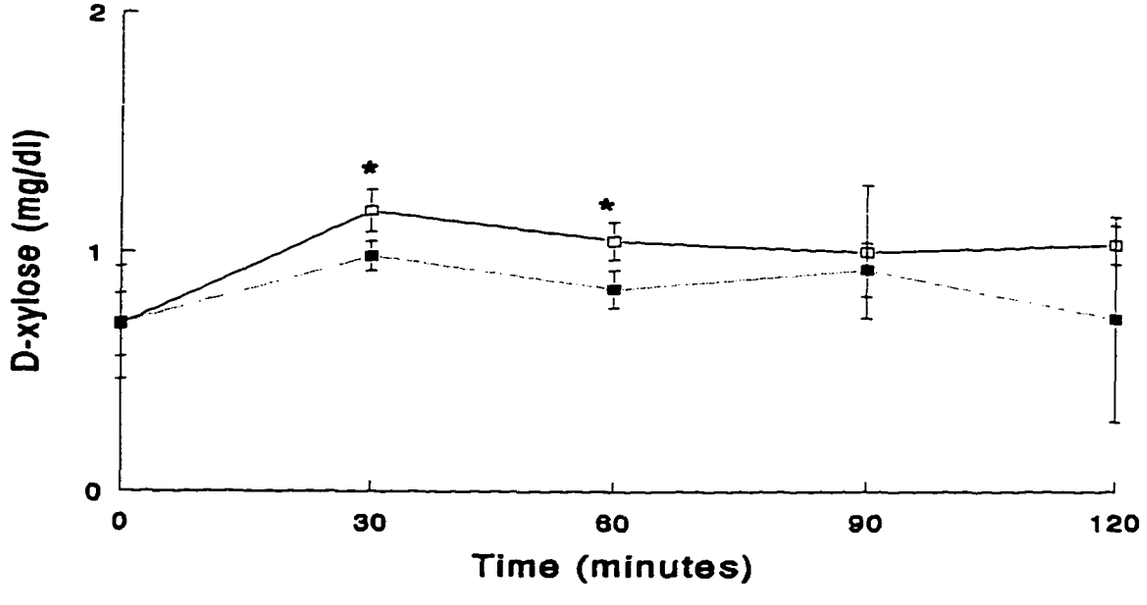


Fig. 3. D-xylose absorption (mg/dl) of turkey embryos inoculated with TPB (□) and stunting syndrome agent (■) at different time intervals following D-xylose administration. The observations with asterisk (*) differ at $P \leq 0.05$.

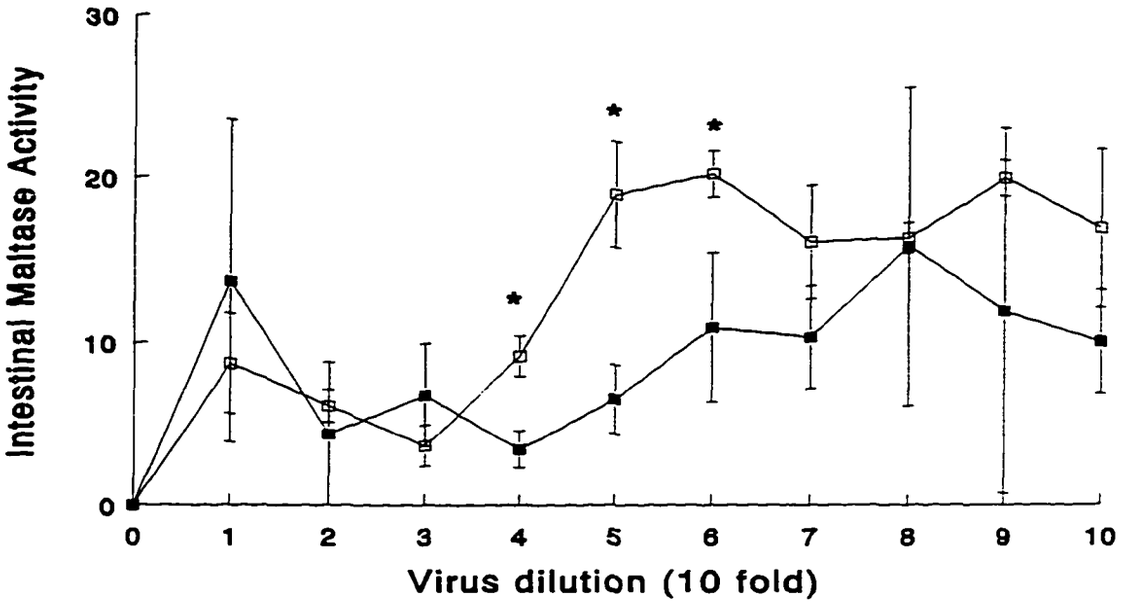


Fig. 4. Specific intestinal maltase activity of turkey embryos inoculated with stunting syndrome agent with (■) and without (□) trypsin activation. The observations with asterisk (*) differ at $P \leq 0.001$.

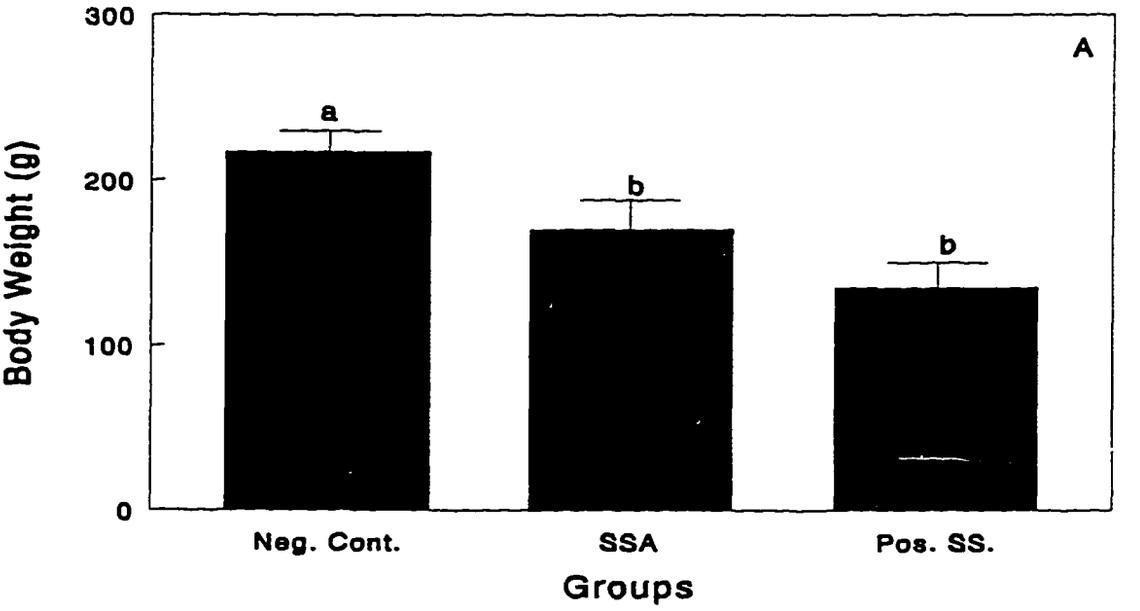
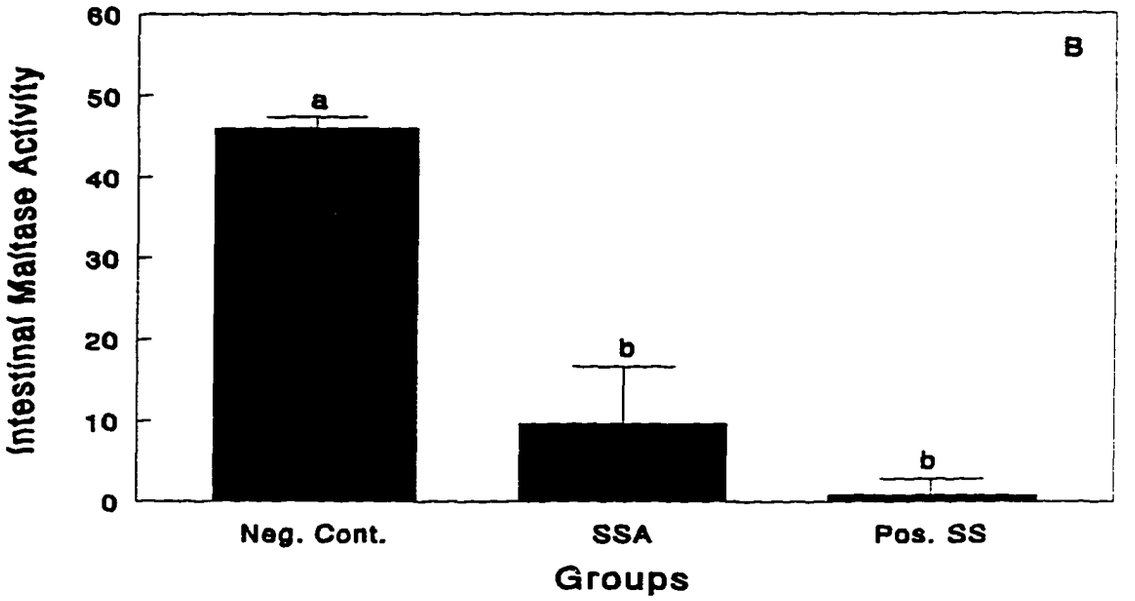


Fig. 5. Body weight (A), specific intestinal maltase activity (B) and D-xylose absorption (mg/dl) (C) of poults inoculated with TPB (Neg. Cont.), embryo passaged stunting syndrome agent (SSA) or positive SS inoculum (Pos. SS) at 9 days PI. Bars with different superscript differ at $P \leq 0.01$.



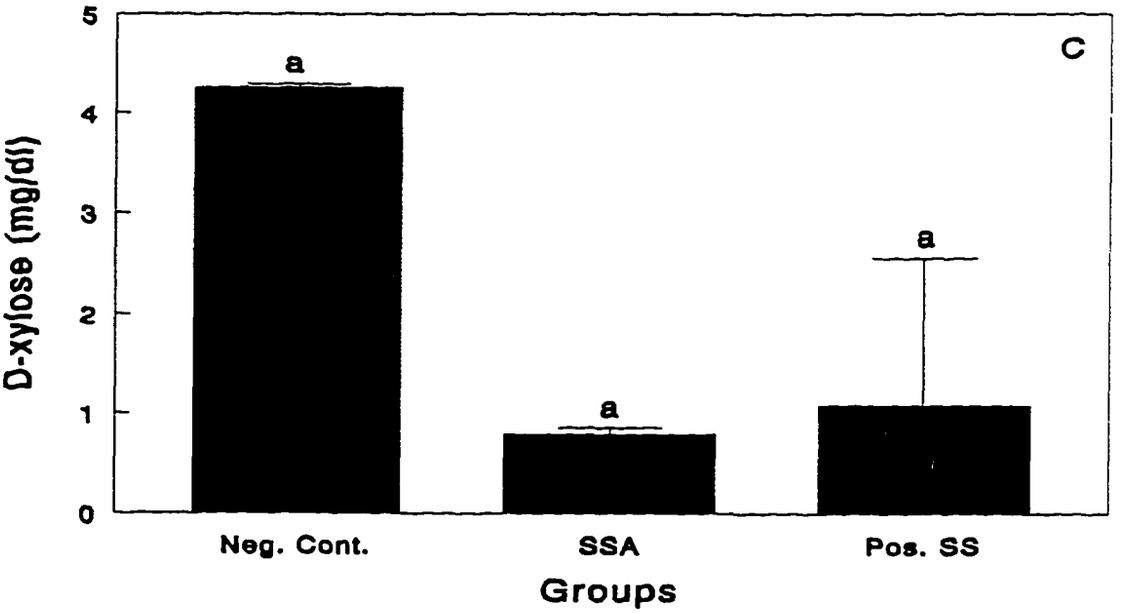


Fig. 5. (Continued)

used for virus propagation were employed in an attempt to propagate SSA. The inoculation of chicken embryos, via different routes, did not result in virus replication on the basis of evaluated parameters (i.e. EM, intestinal lesions and intestinal maltase activity). These observations were in accordance with previous findings that chickens are refractory to SS infection (Dr. Jerry Sell; pers. comm.). The inability of the SSA to propagate in chicken embryos distinguishes it from some turkey coronaviruses (TCV; bluecomb disease agent, Minnesota and Quebec isolates) which have been successfully propagated in chicken embryos (6, 17).

Turkey embryos were also evaluated for their ability to support SSA replication. Infection could not be demonstrated (by EM and lesions) in turkey embryos when inoculated via the chorioallantoic, CAM, and yolk sac route. However, turkey embryos inoculated via the amniotic route resulted in viral replication. The inoculated embryos demonstrated intestinal lesions. The infected intestines were pale, thin-walled and fragile containing pale-yellow to greenish fluid. The amount of fluid varied from 1.5 - 4 ml. The ceca were also markedly distended with this fluid. Similar findings have been reported in chicken and turkey embryos inoculated with TCV (Minnesota strain and Quebec isolates) via the amniotic route (6, 17). It has also been reported that the embryos inoculated with TCV develop gaseous intestinal contents (6). In the present study, some gas accumulation was noted in non-infected control as well as inoculated embryos. This gas accumulation was attributed to bacteria either originating from the embryo or as a result of contamination from the inoculation procedure. The replication of SSA in turkey embryos provides a method for SSA propagation which is less laborious, more convenient and more economically feasible than the primary intestinal epithelial cell culture method used previously.

The infection of turkey embryos by SSA was dependent upon the age of the embryo. Inoculation of turkey embryos via the amniotic cavity at less than 22-days

of age did not result in infection (data not shown). The mechanism of age dependent resistance of embryos, as well as poults (refractory to infection after 3 weeks of age), has not been explored for SSA. The availability of special cells at a particular stage of differentiation, presence of certain cellular receptor(s), physiological condition(s) of the gut environment, maturation status of the immune system, etc., are some of the factors which may be involved in this age dependent infectivity of SSA.

The intestinal function was also affected in embryos inoculated with SSA. There was a significant reduction ($P \leq 0.001$) in intestinal maltase activity in turkey embryos inoculated with SSA via the amniotic cavity. The reduction was similar to poults inoculated with SS. This alteration in intestinal maltase activity of embryos provides a valuable tool for assessing the SSA infection and an objective parameter for evaluating embryo infectivity. The D-xylose absorption was also reduced by the infected embryos similar to SS infected poults. From these observations it was concluded that many of the alterations caused by SSA in embryos are nearly identical to those observed in SS infected poults. Therefore, the embryo can serve as an extraordinary model system for SS infected poults. Such a model system would have distinct advantages for studying the pathophysiologic effects of SSA on the intestinal tract. For example, since the embryo intestine is sterile (or relatively so), the effects can be solely attributed to the agent (i.e., SSA) and not to other factors such as intestinal floral organisms, feed stuffs, etc. The financial resources and the experimental time (only 72 h) of such a model system, when compared to live animal models, are very favorable. Additionally, the embryo model does not meet the criteria for the use of live animals as defined by the current federal regulations. Therefore some ethical issues pertaining to animal usage may be avoided.

Activation of SSA with trypsin resulted in enhanced infectivity for turkey embryos. When SSA was not trypsin activated, even though the trypsin was included in the media, the infectivity was not affected. The requirement for trypsin activation became increasingly essential following embryo passages. Trypsin activation, and/or inclusion of trypsin in the maintenance media, has been reported to enhance the infectivity of many enteric viruses (5, 17, 18, 22) in different cell cultures. The propagation of TCV in cell culture (HRT-18 cells) requires the incorporation of trypsin in the maintenance media (5). While trypsin was not required for initial replication of SSA in embryos, trypsin activation dramatically enhanced viral infectivity. Similarly, it has been reported for a torovirus (Berne virus) that activation with trypsin resulted in enhanced infectivity but inclusion of trypsin in the maintenance media did not (23). The reason why trypsin activation increases infectivity of SSA for turkey embryos is unknown but could be due to low trypsin (or other proteolytic enzymes) activity in the embryonic intestinal tract.

The propagation of SSA in different primary and continuous cells was not successful even when various treatments were employed. The treatments were selected on the basis of previous reports for isolation of various enteric viruses (5, 14, 17, 22). Some TCV isolates have been propagated in turkey embryos and in HRT-18 cells. Our attempts to propagate SSA in HRT-18 cells were not successful (5). Toroviruses, with exception of Berne virus, has not been successfully propagated *in vitro* (10).

In summary, the SSA was successfully propagated in 24-to-25-day-old turkey embryos inoculated via the amniotic route. The infection in embryos resulted in alterations (diarrhea, decreased intestinal maltase activity and decreased D-xylose absorption) very similar to poults inoculated with SS. The attempts to propagate SSA in continuous and primary cells (except primary IECs cell culture) were unsuccessful. In conclusion, the turkey embryos provide a vehicle for *in vitro*

propagation of SSA and a model for studying the pathology, pathophysiology, etc., of SSA and potentially other enteric pathogens.

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CHAPTER 6. CHARACTERIZATION OF THE STUNTING SYNDROME AGENT: RELATEDNESS TO KNOWN VIRUSES

A paper submitted to Avian Diseases

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Summary. An enteric disease of young turkeys, referred to as stunting syndrome (SS), causes reduced growth, impaired feed efficiency and variable mortality. A recently isolated virus (SS agent; SSA) has been found to be the etiologic agent of SS. The objectives of the present study were to determine relatedness of SSA with other viral agents. Serologic (viral neutralization & DAB-ELISA) and reverse transcription-polymerase chain reaction (RT-PCR) were used to determine the relatedness of SSA with other viral agents. The homologous (anti-SSA) antiserum as well as antisera against turkey enteric coronavirus (TCV; bluecomb agent), bovine coronavirus (BCV), bovine breda-1 virus, bovine breda-2 virus, avian infectious bronchitis virus (IBV), avian influenza (AI) virus, Newcastle disease virus (NDV) and transmissible gastroenteritis virus (TGEV) of swine were evaluated by a solid phase, avidin-biotin enhanced DAB-ELISA and by a serum virus neutralization (SVN) assay. The SVN was performed in 24-to-25-day-old turkey embryos inoculated by the amniotic route. The embryo infectivity was assessed on the basis of gross intestinal lesions and intestinal maltase activity at 48 - 72 h post inoculation. The RT-PCR was performed using known specific primers against NDV, IBV, BCV, and TGEV. There was no cross reactivity with any of the known antisera on ELISA. Only the homologous antiserum neutralized the infectivity of SSA in embryos. Results of the RT-PCR revealed that the known primers failed to amplify SSA genome but amplified their respective viral genome. It was concluded that the SSA was distinct from the viral agents that were evaluated.

Keywords. Stunting syndrome, stunting syndrome agent, serum virus neutralization, turkey embryos, ELISA, RT-PCR.

Abbreviations. SS = stunting syndrome, SSA = stunting syndrome agent, PCR = polymerase chain reaction, RT-PCR = reverse transcription-polymerase chain reaction, ELISA = enzyme linked immunosorbant assay, IBV = infectious bronchitis virus, NDV = Newcastle disease virus, AI = avian influenza, BCV = bovine coronavirus, TGEV = transmissible gastroenteritis virus, SVN = serum virus neutralization, DAB-ELISA = dot immunobinding Avidin-Biotin enhanced ELISA.

INTRODUCTION

Stunting syndrome (SS) is a disease of young turkeys that causes diarrhea, depression, reduced weight gain, poor feed conversion, high morbidity and variable mortality (6, 16, 17). A newly identified virus (termed stunting syndrome agent; SSA) has recently been reported to cause SS in turkey poults (4). Inoculation of susceptible poults with purified SSA resulted in poor growth, diarrhea, malabsorption and maldigestion (decreased intestinal maltase activity). SSA has been successfully propagated in turkey embryos inoculated via the amniotic cavity at 24-to-25-days of incubation (5). Inoculated embryos exhibit intestinal fluid accumulation, low intestinal maltase activity and decreased D-xylose absorption. The embryo propagation of SSA provides a method for serum virus neutralization (SVN).

Serologic and genomic based assays are commonly used for detecting and identifying viruses. Serologic assays such as enzyme linked immunosorbant assays (ELISA), SVN, immunofluorescent antibody assay (IFA), immune electron microscopy (IEM) and agar-gel immunodiffusion are widely used for viral diagnosis (7, 13, 15, 19). These assays also provide information useful for virus identification and classification. Polymerase chain reaction (PCR) and/or RT-PCR and hybridization techniques are becoming more widely used for the routine detection of

some infectious disease agents (15, 18, 21, 23). A combination of these two techniques, serology and genome amplification, can be useful for the identification of pathogens.

The objectives of the present study were to demonstrate relatedness of SSA to known viruses using serologic (ELISA and SVN) and genome amplification (RT-PCR) techniques.

MATERIALS AND METHODS

Eggs and embryos. Turkey eggs were procured from a commercial source. Specific pathogen free (SPF) chicken eggs were acquired form Hy-Vac (Hy-Vac Laboratory Eggs Co., Adel IA). The eggs were incubated under standard incubation conditions.

Viruses. SSA was propagated in 24-to-25-days old turkey embryos following a previously described procedure (5). Briefly, the embryos were inoculated with trypsin activated SSA (see below) via the amniotic route. Seventy-two hours post inoculation, the intestinal fluid was harvested from infected embryos. The fluid was clarified by low speed centrifugation (20,000 x *g* for 30 min at 4 C) and ultracentrifuged through a 15% sucrose cushion (200, 000 x *g* for 3 hr at 4 C). The pellet was subjected to density gradient ultracentrifugation using Accudenz® following a previously described procedure (4). The fraction containing virus (visible band) was harvested, dialyzed and concentrated by Centriprep-50 concentrators (Amicon Inc., Beverly, MA).

Infectious bronchitis virus (IBV; Massachusetts vaccinal strain; Solvay Animal Health Inc., Mendota Heights, MN) and Newcastle disease virus (NDV; B1 vaccinal strain, LaSota type; Solvay Animal Health Inc.) were propagated in SPF chicken eggs after inoculation through the allantoic route (2, 11). The viruses were purified on sucrose gradient (3, 8). Bovine coronavirus (BCV; Nebraska Calf diarrhea virus strain; National Veterinary Services Laboratories, NVSL, Ames, IA) was propagated

in HRT-18 cells following a described procedure (10). Subsequently, the virus was purified on a sucrose gradient. Purified TGEV (Miller strain) was kindly provided by Dr. Prem Paul (Veterinary Medical Research Institute, Ames, IA).

Antisera. The antisera to SSA, IBV and NDV were raised in SPF chickens. The purified (see above) viruses (100 µg/ml protein) were emulsified in Freund's incomplete adjuvant (50:50 adjuvant to aqueous virus preparation, Difco Laboratories, Detroit, MI). The emulsified preparations were injected (1 ml/bird) subcutaneously. The injection was repeated after 2 weeks and boostered again at 3 weeks following 2nd injection. Birds were bled 2 weeks after 2nd booster. Antisera to Breda-1 and Breda-2 virus were graciously provided by Dr. G. N. Woode (Texas A & M University, College Station, TX). The antisera were prepared in calves. Antisera to BCV and avian influenza (AI) were procured from NVSL. Antisera to BCV were also raised in turkeys following the same procedure (as above). Antiserum to TGEV (raised in mice) was kindly provided by Dr. Prem Paul (VMRI). Antisera to TCV was generously provided by Dr. Tom Bryan and Tom Hooper (Purdue University, Purdue, IN). This anti-TCV antiserum is routinely used in their diagnostic laboratory in immunofluorescence assay for diagnosis of TCV. This antiserum was prepared in turkeys against a field isolate of TCV from poults experiencing "poult enteritis". The isolation and propagation of this isolate was made in turkey embryos via the amniotic cavity. It also bears antigenic relatedness to Bluecomb disease agent (Minnesota isolate; ATCC # VR-911). All the sera were kept frozen (-80 C) until use. Before use, the sera were thawed, heat inactivated at 56 C for 30 min. and clarified by centrifugation (20,000 x g for 20 min. at 4 C). The sera were filter (0.2 µm) sterilized before using in both assays.

DAB Assay. One-way dot-immunobinding assay enhanced with avidin-biotin ELISA (DAB-ELISA) was performed following a described procedure (9). Briefly, purified SSA (following density gradient ultracentrifugation) was adjusted to a protein

concentration (Bio-Rad protein micro-assay; Bio-Rad Laboratories, Hercules, CA) of 150 µg/ml. One microliter of this diluted virus was adsorbed onto nitrocellulose membranes (0.2 µm pore size; Bio-Rad) placed into the wells of 96-well microtiter plate. The membranes were allowed to air dry at 37 C. The membranes were blocked with 5% nonfat dry milk in tris-buffered saline (TBS; tris 20 mM, NaCl 0.5 M; pH 7.5) and were probed with sera against different viruses. Bound primary antibodies were detected by sequential incubation with respective antispecies biotinylated antibodies (Vector Laboratories, Burlingame, CA and Sigma Chem. Co., St. Louis, MO) appropriately diluted in diluent (TBS with 0.3% BSA). The bound secondary antibodies were detected by incubating in avidin-peroxidase solution (Avidin-D-Horseradish peroxidase; Vector Lab.) made in diluent. The membranes were developed with the developing solution. The developing chromogen solution was prepared as stock (0.3% solution of 4-chloro-1-naphthol in absolute methanol). Immediately before use, 2 ml of this stock solution was diluted with 10 ml of TBS containing 6 µl of 30% H_2O_2. The reaction was stopped by washing with ddH_2O. The positive reaction was indicated by the development of a blue dot at the site of antigen deposition.

Serum Virus Neutralization (SVN) test. SVN test was performed in embryonated turkey eggs (24-to-25 days) following the described procedures (7, 5). The virus (SSA) was titrated (serial 10-fold dilution in tryptose phosphate broth) in turkey embryos to determine the EID_{50}. The parameters of evaluation were intestinal lesions and intestinal maltase activity as described previously (5). The SSA viral suspension used had a titer of $10^{6.3}$ EID_{50}/ml. Appropriately diluted SSA was mixed with equal volumes of undiluted and diluted (two fold) antisera. The mixture was incubated at 4 C overnight. A 0.2 ml of the mixture was injected into the amniotic cavity of 24-to-25-day old turkey embryos as described previously (5). There were 5 eggs per dilution of the sera. Embryonated eggs inoculated with TPB and diluted

virus (EID_{50}) served as negative and positive control respectively. Eggs inoculated with undiluted sera were also included as negative controls. The eggs were incubated under standard hatching conditions. At 72 h post inoculation, the embryos were examined for intestinal lesions and intestinal samples were collected for intestinal maltase activity measurement as described previously (5). The reciprocal of the highest dilution of the serum that inhibited virus replication was considered as the titer.

RT-PCR using known primers. The RNA was isolated from SSA, NDV, IBV, BCV and TGEV using QIAamp Viral RNA extraction kit (Qiagen Inc., Santa Clarita, CA). The primers sequences were obtained from previously published reports for NDV (14, 20), IBV (1), BCV (22) and TGEV (primer sequence provided by Dr. Prem Paul, VMRI). The primers sequences are listed in table 1. The primers were synthesized at DNA synthesis and sequencing facility, Iowa State University, Ames, IA. The RT-PCR was performed using GeneAmp® RNA PCR Core kit (Perkin Elmer Co., Foster City, CA) with annealing and extension conditions from previously described procedures.

RESULTS

DAB assay. The results of DAB-ELISA are shown in table 2. The antisera to known viruses did not cross react with SSA antigen on one-way ELISA. Only the homologous SSA antiserum reacted with SSA.

Serum virus neutralization (SVN) assay. The results of SVN assay are presented in table 2. The antisera to known viruses did not neutralize SSA. The homologous SSA antiserum neutralized the infectivity of SSA in turkey embryos.

RT-PCR. The use of primers for known viruses did not result in amplification of SSA genome under conditions which amplified some part of their respective viral genome. The results are presented in table 3.

Table 1. Sequence of primers for different genes used for amplification of SSA genome.

Virus	Gene	Sequence	Reference(s)
Newcastle disease virus	F	5'-GGAGGATGTTGGCAGCATT-3	14,20
		5'-GTCAACATATACACCTCATC-3'	
	F	5'-CTTTGCTCACCCCCCTTGG-3'	14, 20
		5'-CTTCCCAACTGCCACTGC-3'	
Infectious bronchitis virus	M	5'-TCAGTGGCTTGCTAAGTGTGAACC-3'	1
		5'-TCAAGATGCCCCAACGAGACA-3'	
	N	5'-ACCCTTACCAGCAACCC-3'	1
		5'-GTCTTGTCCCGCGTGTA-3'	
	S	5'-TGAAAACTGAACAAAAGACA-3'	1
		5'-CCTACTAATTTACCACCAGA-3'	
Bovine coronavirus	M	5'-GGGGGATCCTTACACCAGAGGTAGGGGTTC-3'	10
		5'-GGAAGCTTATGGCATCCTTAAGTGGGCCG-3'	
	N	5'-GAACATTTCTAGATTGGTCGGACTG-3'	10
		5'-ATGAGTAGTGTAACTACACCAGCA-3'	
Swine transmissible gastroenteritis virus	S	5'-GTAAAAACATTAGCCACATA-3'	Dr. Prem Paul
		5'-AGGGTAAGTTGCTCATTAG-3'	
	N	5'-GCAACAATCCAATAACAAGAAGG-3'	Dr. Prem Paul
		5'-ACCTCATCAATCATCTCAACCTG-3'	

Table 2. The DAB-ELISA and serum virus neutralization titers of different antisera to stunting syndrome agent.

Antisera	DAB-ELISA titer	Serum virus neutralization titer
Infectious bronchitis virus	≤100	≤2
Newcastle disease virus	≤100	≤2
Avian Influenza virus	≤100	≤2
Bovine coronavirus	≤100	≤2
Turkey coronavirus	≤100	≤2
Swine transmissible gastroenteritis virus	≤100	≤2
Bovine breda-1 virus	≤100	≤2
Bovine breda-2 virus	≤100	≤2
Stunting syndrome agent	≥20,480	≥128

Table 3. RT-PCR of stunting syndrome agent (SSA) using known viral primers.

Viruses/genes	Homologous virus	SSA
Newcastle disease virus		
F gene	+	-
F gene	+	-
Infectious bronchitis virus		
M gene	+	-
N gene	+	-
S gene	+	-
Bovine coronavirus		
M gene	+	-
N gene	+	-
Swine transmissible gastroenteritis virus		
S gene	+	-
N gene	+	-

[1] stunting syndrome agent

DISCUSSION

Stunting syndrome is a disease of young poults causing enteritis, diarrhea, decreased weight gain, etc. (6, 16, 17). Until recently, the etiology of this disease was not known, although, a number of viruses were incriminated as being the causative agents. Recently a virus, referred to as stunting syndrome agent (SSA), was isolated from SS affected poults (4). The SSA is a pleomorphic, membraned virus ranging in size from 60-95 nm in diameter. Inoculation of susceptible poults with gradient purified SSA resulted in decreased weight gain, diarrhea and maldigestion (decreased intestinal maltase activity). The SS was propagated in turkey embryos inoculated through the amniotic route (5). The SSA infection in embryos resulted in diarrhea (fluid accumulation in the intestine), maldigestion (decreased intestinal maltase activity) and malabsorption (decreased D-xylose absorption). These alterations in turkey embryos were similar to SS infected poults. Once Koch's postulates were fulfilled and an *in vitro* system for its propagation was established, attempts were made to establish its relatedness with known, morphologically similar avian and mammalian viruses. The relatedness of SSA with different avian and mammalian viruses was studied on the basis of DAB-ELISA, SVN and RT-PCR. One way DAB-ELISA and SVN assays were used in which SSA virus was reacted with antisera of known viruses. The viruses included in this study were elected on the basis of morphologic resemblance with SSA. The results indicated that only the homologous (anti-SSA antiserum) antiserum reacted with SSA. The heterologous antisera failed to react (one way) with SSA on DAB-ELISA. The results of the SVN assay were similar and indicated that SSA is distinct from the other viruses used in this study. It is not uncommon for a morphologically similar virus to be different antigenically. For example IBV is morphologically similar to other members of the family coronaviridae, but are different antigenically (IBV is placed in a separate antigenic group) (12). Moreover one-way serological tests were

used for detecting relatedness in the present studies. Two-way SVN could not be used because of the inability of other viruses to be adapted to grow through the specific route (amniotic route) and availability of live virus (e.g., Bovine breda viruses, avian influenza viruses etc.). A two-way DAB-ELISA test was also performed for IBV, BCV, NDV, and TGEV (data not shown), however, this was not done with AI virus, bovine breda-1 virus and bovine breda-2 virus. The anti-SSA antiserum did not react with the AI viral antigen (supplied by the NVSL) in an agar gel immunodiffusion test (data not shown).

The primers for known viruses were used to amplify the SSA genome. The primers were selected from those viral genes which are relatively conserved among different members of viral groups. The genes encoding nucleoprotein (NP) and matrix (M) proteins (nonstructural proteins) are generally conserved amongst different members of a virus group. The primers for IBV, NDV, BCV and TGEV amplified their respective viral genome but failed to amplify SSA genome.

In summary, it was found that SSA is antigenically different from TCV, BCV, NDV, AI, IBV, TGEV, bovine breda-1 and bovine breda-2 viruses. It was also observed that SSA genome could not be amplified by using specific known primers for NDV, TGEV, BCV and IBV. The SSA, therefore, appears to be different antigenically and genetically from these viruses. Further studies are needed for definitely identifying and classifying the SSA into the appropriate viral taxon.

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CHAPTER 7. CHARACTERIZATION OF THE STUNTING SYNDROME AGENT: PHYSICO-CHEMICAL PROPERTIES

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Summary. Stunting syndrome (SS) is an enteric disease of turkeys causing diarrhea, reduced weight gain, poor feed efficiency and maldigestion. The etiologic agent is a newly identified, but unclassified virus termed stunting syndrome agent (SSA). The SSA is a pleomorphic, membrane bound virus ranging in size from 60-95 nm in diameter. The objectives of present study were to characterize the physico-chemical properties of SSA. The hemagglutination study revealed that SSA hemagglutinated rat erythrocytes at 4 C and room temperature. Treatment of SSA with ether resulted in loss of infectivity. SSA was resistant to pH changes between pH 3.0 and pH 9.0 at 37 C for 1 h. The virus was inactivated at pH >10. SSA was resistant to treatment with trypsin, chymotrypsin, pancreatin, phospholipase C and sodium deoxycholate. Treatment of SSA with trypsin, chymotrypsin, and pancreatin resulted in enhanced viral infectivity. The viral genome was extracted from purified SSA and was sensitive to RNAse treatment. Using oligo $d(T)_{16-18}$ and random hexamers as primers, the SSA genome was amplified using the reverse transcription-polymerase chain reaction (RT-PCR) conditions but was not amplified using PCR conditions. The enrichment of viral genome was achieved following poly-A^+ selection. These studies provide evidence that the SSA is a positive sense, single stranded RNA virus having many characteristics (stability at acidic pH, resistant to proteolytic enzymes and bile salt) consistent with other membraned enteric viruses.

Key words. Stunting syndrome agent, stunting syndrome, physico-chemical properties, viral genome, hemagglutination, poult enteritis, turkey viral enteritis.

Abbreviations. SSA = stunting syndrome agent, SS = stunting syndrome, IBV = infectious bronchitis virus, DOC = sodium deoxycholate, HA = hemagglutination, DNA = deoxyribonucleic acid, RNA = ribonucleic acid, BSA= bovine serum albumin, SDS = sodium dodecyl sulfate, CDTA = trans-1,2-Diaminocyclohexane-N,N,N',N'-tetraacetic acid, PCR = polymerase chain reaction, RT-PCR = reverse transcription-polymerase chain reaction; EDTA = ethylenediaminetetraaceitic acid, EID_{50} = embryo infective dose 50%, ELD_{50} = embryo lethal dose 50%, ILT = Infectious laryngotracheitis.

INTRODUCTION

Stunting Syndrome (SS) is an infectious disease of young turkeys that results in reduced weight gain, poor feed efficiency, diarrhea, malabsorption, and maldigestion (16, 18, 19). A newly identified virus, stunting syndrome agent (SSA), has been isolated from SS infected poults (3). The virus is a pleomorphic, membraned virus ranging in size from 60 to 95 nm. Inoculation of poults with purified SSA resulted in reduced weight gain, diarrhea, maldigestion and malabsorption. Turkey embryos, inoculated via the amniotic cavity, have been used to propagate SSA (4). SSA infection in embryos results in enteric lesions, maldigestion, and malabsorption. Specific maltase activity of the intestine is used as a parameter for evaluating digestion/maldigestion. Poults inoculated with SS and SSA have decreased intestinal maltase activity (3, 4, 7). Similarly, the intestinal maltase activity decreases when embryos are inoculated with SSA (3, 4). Intestinal maltase activity of the embryos can be used as an objective parameter for evaluating SSA infection.

The family *Coronaviridae* has been divided into two genera; coronavirus and torovirus (17). Coronaviruses infect many avian and mammalian species. Examples include avian infectious bronchitis virus (IBV), bovine coronavirus (BCV), porcine transmissible gastroenteritis virus (TGEV), turkey coronavirus (TCV), etc. The

members of torovirus genus have been reported to cause enteric disease in equine (Berne virus) and bovine (Breda-1 and Breda-2 virus).

The SSA virus has been shown to be antigenically unrelated to many known avian viruses including Newcastle disease virus (NDV), avian influenza (AI) virus, IBV, and TCV. Similarly, SSA did not display antigenic relatedness to the following mammalian viruses: BCV, TGEV of swine, bovine breda-1 virus, and bovine breda-2 virus. Additionally the use of known primers for NDV, IBV, TCV, BCV, and TGEV did not result in SSA genome amplification using the reverse transcription-polymerase chain reaction (RT-PCR).

The objectives of this study were to characterize the physico-chemical properties of SSA. The information gained from this study will furnish insight into the proper classification of this newly identified viral agent.

MATERIAL AND METHODS

Eggs and Embryos. Turkey eggs were procured from a commercial source. Specific pathogen free (SPF) chicken eggs were acquired from Hy-Vac (Hy-Vac Laboratory Eggs Co., Adel, IA). The eggs were incubated under standard incubation conditions until used.

Stunting syndrome agent Virus. SSA was propagated in turkey embryos and purified from the intestinal fluid following previously described procedures (3, 4). Briefly, 24-to-25-day-old turkey embryos were inoculated via the amniotic cavity with trypsin activated SSA (see below). Seventy-two hours post inoculation, the gut fluid (containing virus) was harvested and purified on Accudenz® (Accurate Chemical & Scientific Co., Westbury, NY) as described previously (3). The virus was concentrated using concentrators (Centriprep-50; Amicon Inc., Beverly, CA) to avoid virus aggregation as a result of pelleting.

Trypsin activation of virus. Purified virus was trypsin activated (20 µg type IX trypsin; Sigma Chemical Co., St. Louis, MO) at 37 C for 1 h. The trypsin activity was

neutralized by soybean trypsin inhibitors (Sigma) following the manufacturer's instructions. The purified, trypsin activated SSA was aliquoted and stored in TNEM buffer (tris-HCl 10 mM, NaCl 100 mM, $MgCl_2$ 2 mM, and EDTA 1 mM; pH 7.0) at -80 C until use. Prior to storage (-70 C), viral titer was determined by titration in turkey embryos as described below.

Infectious bronchitis virus. Infectious bronchitis virus (IBV; Massachusetts vaccinal strain; Solvay Animal health Inc., Mendota Heights, MN) was propagated in SPF chicken embryos by chorioallantoic route inoculation (1, 2). The virus was purified from the chorioallantoic fluid by sucrose gradient ultracentrifugation following a previously described procedure (1, 2). The virus was stored at -80 C until use.

SSA infectivity/titration assay. The infectivity titration of SSA was performed by inoculating turkey embryos via the amniotic route as described above. The virus titer (embryo infectious dose-50%; EID_{50}) was determined by injecting 0.2 ml volumes of serially (10-fold) diluted SSA into the amniotic cavity. The infection was assessed on the basis of lesions and intestinal maltase activity (4). The infectivity titers (EID_{50}) were determined using the Spearman-Kaerber formula (20).

IBV infectivity/titration assay. The infectivity titration of IBV was determined in SPF chicken embryos. Serially (10 fold) diluted virus was injected (0.2 ml/embryo) into the chorioallantoic cavity of 9-to-11-day-old chicken embryos. Embryo mortality was used for calculating the infectivity titer (embryo lethal dose-50%; ELD_{50}) by using the Spearman-Kaerber formula.

Thermal sensitivity. Virus suspensions (aliquots of 1 ml) were incubated at temperatures of 35, 40, and 45 C for 1, 2, 5, 10, 15, 20, and 24 hr; at 50, and 55 C for 0.25, 0.5, 0.75, 1, and 2 hr. After incubation, the suspensions were chilled on ice until used. The viral suspensions were diluted for titration in RPMI-1640 medium (Sigma) prior to embryo inoculation.

Virus stability was also evaluated after storage at 4, -20 and -80 C. The virus preparation stored at 4 C were evaluated at 1, 2, and 4 weeks, whereas those stored at -20, and -80 C were evaluated at 1, 3, and 6 months. The infectivity of SSA was determined by inoculating turkey embryos.

pH stability. pH sensitivity was assessed by suspending the virus in citrate-phosphate-borate-HCl buffer (0.1 ml virus suspension and 0.9 ml buffer at a predetermined pH). The final pH of the suspensions were adjusted to 2.5, 3.0, 4.0, 5.0, 7.0, 9.0, 10.0, 11.0 and 13.0 by adding 1N HCl or 1N NaOH. The preparations were incubated for 1 h at 37 C. The suspensions were brought to pH 7.0 by 1N HCl or 1N NaOH before injecting into the embryos.

Ether sensitivity. The sensitivity to ether was determined following a previously described procedure (6). The suspension of SSA was mixed with ethyl ether (4:1 virus:ether mixture) and mixed gently at 4 C for 18-24 h. Intestinal fluid from normal embryos was treated in a like manner and served as a negative control. The mixture was poured into a petri dish and the ether was allowed to evaporate under a biosafety hood. The virus was assayed for infectivity using turkey embryos.

Enzymes sensitivity. Enzyme treatments were performed using non-activated pure virus following a published protocol (21). Bovine trypsin, ß-chymotrypsin and pancreatin were prepared in TES buffer (10 mM tris-HCl, 1 mM EDTA, 150 mM NaCl, pH 7.2) at twice the final concentration. Equal volumes of the enzyme solution and virus suspension were mixed and incubated at 37 C for 1 h. The suspensions were immediately chilled on ice and the enzyme activities were neutralized by protease inhibitors (Complete™ protease inhibitor cocktail tablet ; Boehringer Mannheim, Indianapolis, IN) following the manufacturer's recommendations.

Phospholipase C dissolved in tris buffer (tris-HCl 10 mM, NaCl 100 mM, $MgCl_2$ 1 mM; pH 7.5) was used at a final concentration of 0.15 U/ml. The enzyme-virus mixture was incubated at 4 and 37 C for 1h. The mixture was serially diluted

for titration in RPMI-1640 medium and kept on ice until inoculation. The infectivity was assessed using turkey embryos. Purified IBV was treated in the same way and the infectivity was assessed in SPF chicken embryos.

Detergent treatment. The susceptibility of SSA was determined for sodium deoxycholate (DOC) and triton X-100. A 0.2 % solution (in TES buffer) of DOC was mixed with equal volumes of purified SSA and incubated at room temperature. After 1, 5, 10, 20 and 30 min intervals, samples were withdrawn and serially diluted for titration in RPMI-1640 medium. The infectivity (EID_{50}) was assessed in turkey embryos. Purified IBV was treated in a similar fashion and the infectivity (ELD_{50}) was assessed in SPF chicken embryos.

Triton X-100 was used at final concentrations of 1% and 0.1% in TES buffer. The triton-virus mixture was incubated at room temperature. Samples were withdrawn after varying time intervals (1, 5, 10, 20, 30 min.) and were serially diluted for titration in RPMI-1640 medium. The infectivity (EID_{50}) was assessed in turkey embryos.

Hemagglutination (HA) assay. The hemagglutination assay was performed using erythrocytes from chickens, turkeys, rabbit, guinea pig, rat, mouse, ovine, bovine, canine, equine and feline. The assay was performed following a described protocol (8). Blood was collected in equal volumes of Alsever's solution. The erythrocytes were washed several times in HA buffer (145.5 mM NaCl, 5 mM KH_2PO_4; pH 7.0) containing 0.1 % bovine serum albumin. A 0.5 % suspension of red cells prepared in HA buffer with 0.1 % BSA was used. The assay (two-fold virus dilution) was carried out in round bottom 96-well microtiter plates in 100 µl volumes. Incubation was at room temperature, 4 C or 37 C. The HA titer was determined as the reciprocal of the highest dilution of virus that caused complete hemagglutination.

Viral nucleic acid determination. Viral nucleic acid was extracted and purified using a previously described procedure (9) under DNA and RNA extraction

conditions. For RNA, concentrated preparation of purified virus was mixed with 10x RNA extraction buffer (SDS 5%, NaCl 5M, CDTA 0.1M, tris-HCl 0.5M; pH 7.4 and 1% β-mercaptoethanol added prior to use) and incubated with 100 µg/ml proteinase K at 55 C for 1 h. Purified SSA was mixed with 2x DNA extraction buffer (urea 8.0 M, NaCl 0.4 M, Tris-HCl 0.2 M pH 8.0, 1% n-laurylsarcosine, CDTA 0.02 M) and incubated with 100 µg/ml proteinase K at 55 C for 1h. The nucleic acid was extracted with phenol/chloroform/isoamyl alcohol (phenol pH 8.0 for DNA and 4.5 for RNA) twice and with chloroform/isoamyl alcohol once. The nucleic acid was precipitated with 7.5 M ammonium acetate and ethanol at -80 C overnight.

Two procedures were used to identify the nature of viral nucleic acid.

1. **Enzymatic determination of nucleic acid type.** The nucleic acid was treated with DNAse and RNAse enzymes and electrophoresed under normal and denaturing conditions for DNA and RNA respectively (20). Following electrophoresis, the gels were stained with ethidium bromide and examined under UV light for the integrity of the purified nucleic acid. Nucieic acids extracted from infectious laryngotracheitis virus (ILT; ds DNA), rotavirus (ds RNA), IBV (ss RNA) and parvovirus (ss DNA) were used as controls.

RNAse treatment. The extracted viral genomes were digested with RNAse A enzyme under single stranded (ss) and double stranded (ds) conditions. ssRNA was incubated with 5 µg/ml RNAse A (Promega Co., Madison, WI) in 2x SSC buffer (NaCl 0.1M & sodium citrate 0.015 M) for 30 min at room temperature. dsRNA was digested under similar conditions except 0.1X SSC buffer was used.

DNAse treatment. The extracted viral genomes were incubated with 10 µg/ml DNAse in 10 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$ at 37 C for 30 min. The reaction was stopped by heating at 70 C for 5 min.

The genome extracted from known viruses were also subjected to these enzyme treatments. The genomic nucleic acids were subjected to gel

electrophoresis before and after enzymatic digestion. The gel electrophoresis for RNA and DNA was performed following described procedures (20). For DNA, the electrophoresis was performed in tris-borate-buffer (TBE) using 0.4% agarose gel. For RNA, the formaldehyde-denaturing agarose gel was used. The RNA samples were denatured with RNA sample loading buffer (5 prime -- 3 prime, Inc., Boulder, CO) at 70 C for 10 min followed immediately by chilling on ice for 5 min. Electrophoresis was performed in 0.8 % agarose in MOPS buffer [0.02 M 3-(N-morpholino)propanesulfonic acid, pH 7.0, 8 mM sodium acetate, 1 mM EDTA, pH 8.0] under formaldehyde denaturing conditions. Following electrophoresis the gels were stained with ethidium bromide and examined for the integrity of viral genomes.

2. PCR/RT-PCR method for determination of nucleic acid type. An indirect approach was used to determine the nucleic acid type of SSA. The principles of polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR) were employed. Nucleic acids from ILT (ds DNA), rotavirus (ds RNA), IBV (ss RNA) and parvovirus (ss DNA) were used as controls. Purified nucleic acids were subjected to PCR and RT-PCR using DOP PCR master (Boehringer Mannheim Co., Indianapolis, IN) and RT-PCR Core (Perkin Elmer Applied Biosystems,) kits following the manufacturer's instructions. For PCR, random (hexamers) primers were used. The reverse transcription in RT-PCR reaction was performed using oligo-$dT_{16\text{-}18}$ primer and PCR amplification was performed using the random hexamers. The genomic nucleic acids were subjected to complete digestion with DNAse and RNAse enzymes (as above), extracted with phenol-chloroform-isoamyl alcohol, and subjected to PCR and RT-PCR reactions. The amplified products, after electrophoresis in 1% agarose gels, were stained with ethidium bromide before visualization.

Enrichment of genome for mRNA. The genome isolated form NDV (negative sense ss RNA), IBV (positive sense ss RNA) and SSA was subjected to poly-A^+

selection using oligotex mRNA mini kit (Qiagen Inc., Santa Clarita, CA). RNA isolated following poly-A^+ selection was subjected to RT-PCR as described above. The amplified products were visualized in 1% agarose gel following ethidium bromide staining.

RESULTS

Hemagglutination. The results of hemagglutination test are shown in table 1. The SSA hemagglutinated rat erythrocytes at room temperature and at 4 C. At 37 C, the hemagglutination was more rapid than at room temperature followed by elution within 1 h of incubation. SSA did not hemagglutinate erythrocytes from other species that were tested.

Ether Sensitivity. Treatment of SSA with ether resulted in a sharp reduction in the infectivity. The results are presented in table 2.

Thermal Sensitivity. The results of heat inactivation on purified SSA are shown in fig. 1. The titer of the initial preparation was $10^{6.6}$ EID_{50} /ml. The virus was stable at 50 C for 1 h. The virus inactivation proceeded in a linear fashion at 45 and 40 C.

Purified virus stored at -20 C lost its infectivity rapidly after 3 months but was stable after six months at -80 C. At 4 C, the loss of infectivity depended on the presence of buffer. SSA stored in sterile water lost its infectivity in <10 days. Whereas, the SSA in TNEM buffer was infectious for the turkey embryos after two weeks storage at 4 C. The infectivity reduced dramatically after 4 weeks storage at 4 C.

pH Sensitivity. The infectivity of SSA was unaffected between pH 3.0 and 9.0. The infectivity declined after pH 9.0 and inactivation was complete at pH >10. Infectivity was slightly reduced at pH 2.5 but inactivation of SSA occurred at pH <2.5. The results are shown in fig. 2.

Sensitivity to enzymes. The SSA infectivity was not altered significantly by treatment with trypsin, ß-chymotrypsin and pancreatin at all the concentrations

Table 1. Hemagglutination profile of SSA at different temperatures.

Source of RBCs	HA at different temperatures		
	4	22[1]	37
Chickens	-[2]	-	-
Turkeys	-	-	-
Rabbit	-	-	-
Mouse	-	-	-
Rat	+[3]	+	+/-[4]
Guinea Pig	-	-	-
Feline	-	-	-
Equine	-	-	-
Canine	-	-	-
Ovine	-	-	-
Bovine	-	-	-

[1] room temperature.
[2] no agglutination.
[3] agglutination.
[4] agglutination and elution.

Table 2. Sensitivity of SSA to ether.

Treatment	Virus titer (log_{10} EID_{50}/ml)	
	Before treatment	After treatment
Ethyl ether (4 C for 18-24 h)	6.1	0.5

tested (Fig. 3). These enzymes caused an increase in infectivity of SSA. Treatment of SSA with phospholipase C did not demonstrably affect the infectivity of SSA at 4 and 37 C. The titer of SSA declined from $10^{5.5}$ to $10^{4.6}$ at 4 C after 1 h. At 37 C, the loss in infectivity of SSA was from $10^{5.5}$ to $10^{4.2}$ EID_{50}/ml. Treatment of IBV with the enzyme phospholipase C resulted in loss of infectivity from $10^{5.2}$ to $10^{1.6}$ ELD_{50}/ml infectivity for chicken embryos after 1 h at 37 C.

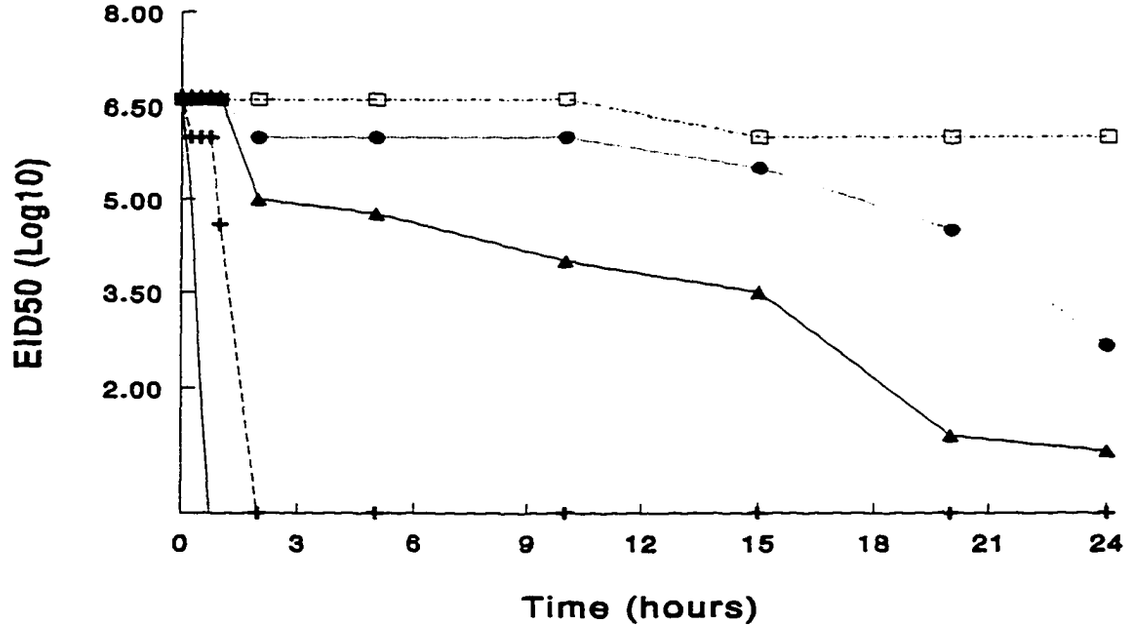

Fig. 1. The EID_{50} (log_{10}) of turkey embryos inoculated with stunting syndrome agent following heat treatment at 55 C ( ), 50 C (+), 45 C (▼), 40 C (●) and 35 C (□).

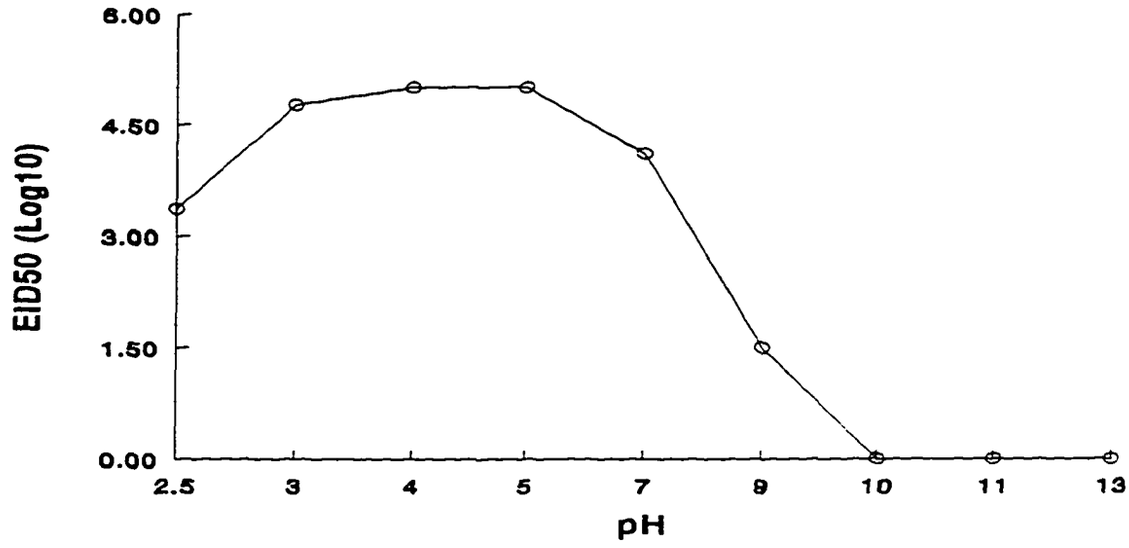

Fig. 2. The EID_{50} (log_{10}) of turkey embryos inoculated with stunting syndrome agent following treatment at different pH.

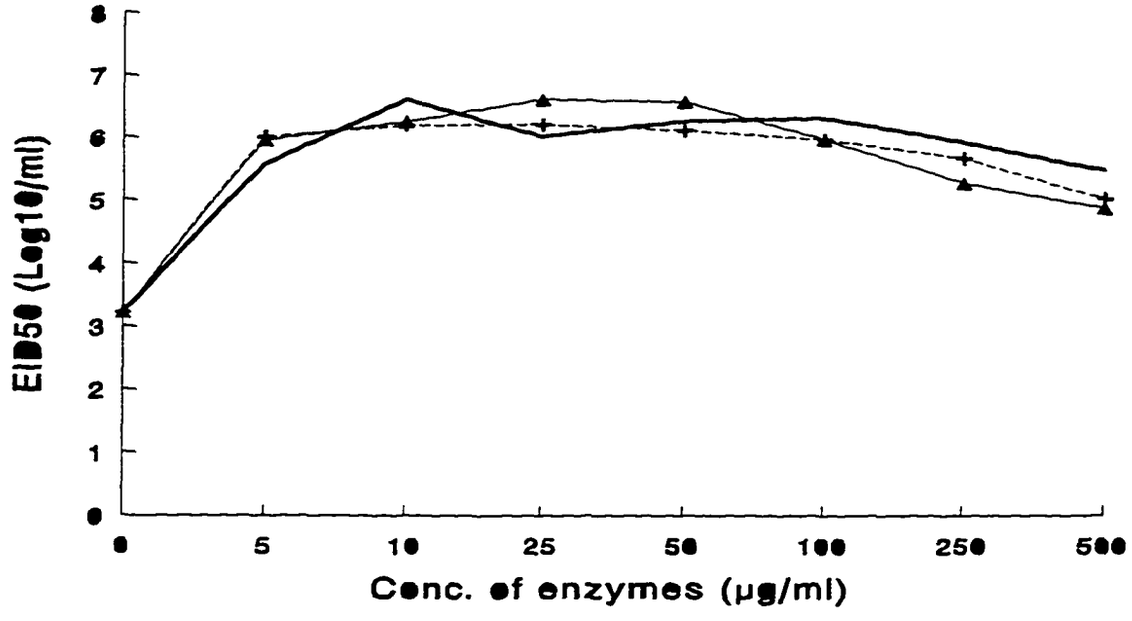

Fig. 3. The EID_{50} (log_{10}) of turkey embryos inoculated with stunting syndrome agent following treatment with trypsin ( ), chymotrypsin (+) and pancreatin (▼).

Table 3. Treatment of viruses with sodium deoxycholate and triton-x.

Treat.	Conc.	Viruses	Log_{10} titer (before treat.)	Log_{10} titer (EID_{50}/ml) after 1	5	10	20	30 (min)
DOC[1]	0.1%	SSA[2]	6.6[4]	6.5	6.0	6.1	5.6	5.1
	0.1%	IBV[3]	5.2[5]	ND[6]	ND	ND	ND	0.25[5]
Triton-x	0.1%	SSA	6.6	3.6	2.6	3.2	2.6	3.2
	1.0%	SSA	6.6	3.7	2.1	2.2	2.7	1.5

[1] Sodium deoxycholate.
[2] Stunting syndrome agent.
[3] Infectious bronchitis virus.
[4] ELD_{50}/ml
[5] EID_{50}/ml.
[6] Not done

Detergent treatment. Treatment of SSA with DOC did not result in appreciable loss of infectivity at different time intervals (table 3). Whereas the infectivity of IBV was abolished by treatments with DOC after 30 min. Treatment of SSA with 0.1 and 1.0 % triton-x resulted in detectable loss of infectivity for turkey embryos. This loss occurred immediately after the addition of triton-x and was independent of time. The results are presented in table 3.

Type of viral genome. Digestion with DNAse did not result in SSA genome fragmentation. However, treatment with RNAse resulted in complete digestion of the genome on the basis of ethidium bromide stained agarose gel. The results of the PCR/RT-PCR are shown in table 4 & 5. Direct PCR using random hexamers, did not result in genome amplification of SSA, however, the genome of ILT was amplified. The reverse transcription of the SSA genome with reverse transcriptase and then amplification of the cDNA using random hexamers did generate amplified products. Similarly, the RT-PCR of the IBV genome generated amplified products. Complete digestion of SSA genome with RNAse resulted in no amplified products on ethidium bromide stained gels.

Genome enrichment of IBV and SSA after poly A selection gave amplified products following RT-PCR. The enriched genome of NDV did not result in detectable amplified products following RT-PCR.

DISCUSSION

Stunting syndrome, an enteric disease of young poultry, has recently been reported to be caused by a newly isolated virus termed stunting syndrome agent (SSA). SSA is a pleomorphic membraned virus ranging in size from 60-95 nm in diameter (3). This newly identified virus is unrelated to many known avian and mammalian viruses both antigenically and genetically (5). Therefore proper classification of this viral agent has not been proposed. The present study was

Table. 4. Results of PCR/RT-PCR using oligo d(T) and random hexamer primers.

Viruses	Type of Genome	PCR / RT-PCR results	
		PCR	RT-PCR
SSA[1]	?	-	+
IBV[2]	[3]ssRNA	-	+
ILT[4]	[5]dsDNA	+	+
Rotavirus	dsRNA	-	-
Parvovirus	ssDNA	+	+

[1]Stunting syndrome agent
[2]Infectious bronchitis virus
[3]Single stranded
[4]Infectious laryngotracheitis virus
[5]Double stranded

Table. 5. Results of PCR/RT-PCR using oligo d(T) and random hexamer primers after digestion of viral genomes with enzymes.

Viruses	*Type of Genome*	Results after digestion of viral genomes with			
		RNAse		DNAse	
		PCR	RT-PCR	PCR	RT-PCR
SSA[1]	?	-	-	-	+
IBV[2]	ssRNA[3]	-	-	-	+
ILT[4]	dsDNA[5]	+	-	-	-
Rotavirus	dsRNA	[6]ND	ND	ND	ND
Parvovirus	ssDNA	+	+	-	-

[1]Stunting syndrome agent.
[2]Infectious bronchitis virus.
[3]Single stranded.
[4]Infectious laryngotracheitis virus.
[5]Double stranded.
[6]Not done.

undertaken to study biological and various physico-chemical properties of SSA which may help to properly classify this agent in the future.

The SSA only hemagglutinated rat erythrocytes. Hemagglutination was completed within the first hour when performed at room temperature. However, at 4 C, complete hemagglutination required a longer time. At 37 C, hemagglutination occurred swiftly but elution was predominant afterwards. Hemagglutination and elution was complete within an hour of incubation. Coronaviruses of turkeys (TCV; Minnesota and Quebec isolates) cause hemagglutination of erythrocytes from rabbits and guinea pigs (10). Moreover, the hemagglutination by SSA occurred without any enzymatic treatment of the virions as is required for IBV. Many strains of IBV does not cause hemagglutination unless treated with the enzyme phospholipase C (14). Bovine breda viruses (torovirus), morphologically similar to SSA, have also been reported to agglutinate only rat erythrocytes (23). The hemagglutination profile and morphological similarities of SSA with bovine Breda virus suggests that SSA may be related to toroviruses.

The loss of infectivity of SSA for turkey embryos after treatment with ethyl ether substantiates the presence of an envelope. Similar observations have been observed with many enteric membranous viruses from different animal species with varying susceptibility to organic solvents (17). Organic solvents completely abolish the infectivity of some enteric enveloped viruses but only partially decrease the infectivity of other.

The SSA is stable over a wide range of pH especially towards the acidic range. Many of the known enveloped viruses such as orthomyxoviruses, paramyxoviruses, rhabdoviruses, retroviruses, and arenaviruses are inactivated at pH values <5.0 and some togaviruses at pH <6.0 (17). Conversely, Berne virus (a torovirus) and enteric coronaviruses (e. g., TCV) are stable at acidic pH (11, 22). This stability at acidic pH is not an uncommon characteristics of many enteric non-

enveloped viruses (12, 17) but less common for many enveloped viruses (see above). This characteristic may be advantageous to enteric viruses since they have to survive the acidic pH of the gastrointestinal tract.

The infectivity of SSA was enhanced by treatment with trypsin, ß-chymotrypsin and pancreatin. It has been demonstrated in our laboratory that SSA infectivity for turkey embryos was enhanced 3-4 fold after activation with trypsin. This enhancement in infectivity following proteolytic enzyme treatment could be either due to dispersion of viral aggregates or due to intrinsic properties of the virions. The latter has been reported for other enteric viruses e.g., Berne virus (23), rotaviruses (13), etc. Furthermore, this resistance to proteolytic enzymes explains its (SSA) adaptation and survival in the gastrointestinal tract.

The SSA has also been found to be resistant to the enzyme phospholipase C and DOC (bile salt). Resistance to phospholipase C enzyme indicates the relative inaccessibility of envelop lipids to the enzyme. This is also a prominent feature of Berne virus (22). On the other hand, the infectivity of IBV was dramatically reduced after treatment with the enzyme phospholipase C. The SSA infectivity was marginally affected by the treatment with DOC. This may also reflect the biological property of this enteric virus to withstand the effects of the bile acids. Conversely, the infectivity of IBV was abolished after treatment with DOC. It appears that the viruses which primarily affect the gastrointestinal system are resistant to the effects of DOC (e.g., Berne virus) and those which target other organ systems (e.g., togavirus, IBV, etc.) are susceptible.

Digestion of SSA genome with RNAse (under single stranded digestion conditions) resulted in its degradation, thus, providing the evidence that SSA has a ss RNA genome. The genome enrichment after selection with oligo d(T) is indicative of a poly-A^+ tail and provide strong evidence for positive sense RNA. Moreover, first strand synthesis using oligo d(T) as a primer in RT- with subsequent amplification

using random hexamer primers (PCR) also suggests a positive sense ssRNA. However, presence of multiple adenine residues in the genome cannot be ruled out. When comparing the two methods used to determine the nature of viral genome, the PCR/RT-PCR method proved easier to perform than the genome visualization method. Visualization of ssRNA in a denaturing gel requires 3 to 4 log more RNA than the PCR/RT-PCR method. Moreover, when using the PCR/RT-PCR method, problems of toxic chemicals (e.g., denaturing components in formaldehyde gel) and the danger of RNAse contamination were reduced to a minimum.

The physico-chemical characteristics of SSA, such as resistance to the proteolytic enzymes trypsin, chymotrypsin, pancreatin etc., stability at low pH, and resistance to DOC (bile slat) treatment are characteristics consistent with many enteric viruses. Such characteristics enable theses viruses to survive in a hostile environment of the gastrointestinal tract. Stability after treatment with DOC and phospholipase distinguishes it from IBV which was sensitive to these treatments. SSA had many similarities with toroviruses including morphology, resistance to acidic pH, proteolytic enzymes, bile salts, phospholipase C and hemagglutination profile. Some of these characteristics are also common with enteric coronaviruses. Further information is needed for proper classification of SSA.

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CHAPTER 8. GENERAL DISCUSSION

The gastrointestinal (GI) tract is an important organ of the body that performs several functions. The primary function of the GI tract is to digest and absorb nutrients. Being an organ with one of the largest surface areas exposed to the environment, it constantly comes in contact with various kinds of insults. Some of these insults result in disease. Maldigestion and malabsorption is not an uncommon outcome of many of these diseases. The important consequences of these outcome in food animals is a reduction of performance. Insults may be either infectious or non-infectious. The infectious agents include viruses, bacteria, fungi, protozoa and/or their combinations. Although, the hostile environment of the gut (pH, bile salts, mucin etc.) protects itself from many infectious agents, some agents have also evolved to evade these protective mechanisms. Of these, the viral agents pose a distinct challenge for diagnosis, treatment and control.

Enteric diseases in poultry are not uncommon and generally result in poor feed efficiency, reduced weight gain and hence poor performance. An enteric disease situation in young poultry is often quite complex largely due to the inability to associate the disease condition(s) with a definitive etiologic agent(s). These enteric disease conditions have been collectively termed the "enteric disease complex" (209). One member of this "enteric disease complex" is stunting syndrome (SS) of turkeys. SS is a disease of turkey poults that occurs during the first 1 - 3 weeks of age. The infection results in diarrhea, enteritis, maldigestion, malabsorption and, hence, poor performance (209). A number of viruses has been found in association with "enteric disease complex" but no one virus, or a combination of viruses, has been confirmed as being the cause. The inability to establish a definitive cause has been complex and challenging. One of the major challenge is the inability to cultivate many of the viruses (that cause GI tract infection) *in vitro*. Although electron microscopy (EM) has been, and is being, used

to identify enteric viruses, it has limitations. Many of the enteric viruses observed under EM are also present in healthy/normal animals (commensal organisms). Additionally, the diseased individuals may contain more than one morphologic type of virus which limits the usefulness of EM for a definitive diagnosis. Furthermore, some viruses may cause disease in other organs/systems and are the passive inhabitants of the GI tract. Sometimes these, and other commensal viruses, are relatively easy to culture, and often are overgrown in tissue culture. Artifacts of the GI tract can be easily confused with viral particles (e.g., debris from sloughed cells can be confused with membranous viruses). The simple presence of a virus and/or viruses, therefore, is not indicative of infection. The sensitivity and specificity of EM and other diagnostic techniques for diagnosing enteric viral infections can often be improved by the use of specific reagents (immune electron microscopy; IEM, PCR, etc.). However, generation of specific reagents often requires pure viral antigen. Sometimes, virus can be obtained from the infected gut contents of the host animals, however, often these techniques have limitations. Experience from the present work indicates that it is extremely difficult to purify a virus (by density gradient ultracentrifugation) from the gut contents without simultaneously purifying some cell membrane debris which may band at or near the same density as the viral particles. In some cases of enteric viral infections seroconversion is very poor, thus, limiting the ability to obtain specific antibodies. Some of these limitations were factors in identifying the etiologic agent of SS.

The etiology of SS in turkey poults has not been previously reported. Scanning electron microscopic examination of the intestines from SS infected poults revealed long segmented filamentous organisms (LSFOs) attached to the enterocytes (4). However, SS inoculum following filtration through 0.2 μm filters produced the disease in susceptible poults (231). It was deduced from filtration studies that the bacteria were not the primary cause for SS. Efforts were focused on

identifying virus(es) as the etiologic agent(s). Initially, conventional direct EM and IEM (using the convalescent sera) were used with fecal material and/or intestinal contents for virus(es) identification. The feces were filtered through 0.2 μm filters and ultracentrifuged. The pellet was examined by EM. Additionally, fecal material from infected poults was blindly passaged in different cell lines (e.g., MA 104, HRT-18, etc., which have been successfully used to isolate other enteric viruses). These efforts proved futile. It was hypothesized that the cells cultured from the same organ (day old poult intestine) would support the growth of the etiologic agent(s). A successful technique was established for the *in vitro* culture of turkey intestinal epithelial cells (IECs). A problem arose as to how to detect the replication of the agent(s) in the cells. A simple, convenient, and most commonly used method for detecting viral replication in cell culture is the presence of CPE (cytopathic effects). CPE have been used not only to monitor viral infection of the cells but also sometimes for their quantitation (e.g., plaque counting in virus neutralization assays, titration assays, etc.). The turkey IECs cultured *in vitro* seldom formed a confluent monolayer. Therefore, the use of CPE in this system was limited. Additionally, recent studies on the histopathology of GI tract following SS infection have shown that the villus architect remains unaltered during SS infection (Dr. Harley Moon; Pers. Comm.). Furthermore, even if the virus(es) grew in cell culture, the question remains as to weather it causes the disease. Moreover, there is also the probability that *in vitro* propagation might alter viral pathogenicity (i.e., attenuation). If the isolated virus did not reproduce the disease, then Koch's postulates would be difficult to fulfill. It was assumed while contriving the above hypothesis (that cultured turkey IECs may be helpful in isolating the etiologic agent of SS) that the IECs are the target cells during SS infection. Since no CPE were observed in cultured IECs (after infection with SS inoculum), it was imperative to prove the hypothesis that indeed IECs are the target cells. It was, therefore, proposed that the IECs purified

from SS infected poults could be used as an inoculum to reproduce SS in susceptible poults. In trial I (chapter 4) the hypothesis that the IECs are the target cells was proved. The viral particles were observed in cell (IECs) lysate filtrate. These particles were termed stunting syndrome agent (SSA). This technique of using the target cells as an inoculum and for virus isolation, identification/purification was very successful and could be used for other applications especially if one needs to isolate infectious agent(s) from a single cell type. Density gradient centrifugation separation techniques has been established for many cell types and can be used for a particular cell type isolation and purification. Other advantages of this method included the absence of fecal contents and other gut microfloral contents.

It was determined that SSA purified from lysed IECs caused disease in susceptible poults (see chapter 4). The infected poults secreted virus into the environment as evidenced by the fact that uninoculated susceptible poults, introduced into previously contaminated isolators, contracted the disease (environmental exposure). The SSA could be recovered from the feces of the environmentally exposed poults as well as from their epithelial cells. The recovery of the virus was much higher from the epithelial cells than from the feces. There was a very short period of time when virus could be recovered from the feces (first 2-4 days post infection). However, the virus titer was so low that polyethylene glycol/ammonium sulfate precipitation was needed to demonstrate the virus (data not shown). The virus that was recovered by this technique displayed few peplomers. It was also noted that SSA purified from samples that were subjected to multiple freeze-thawing cycles had lost peplomers. This was more noticeable when purified SSA was subjected to freeze-thawing cycles. After a single freeze-thaw cycle, the majority of viral particles lost their peplomers and freeze-thawing was found to have a negative impact on viral infectivity. Freeze-thawing is often used during virus isolation and purification techniques from enteric virus samples. This

may attribute to the lack of success when these conventional techniques were used initially for identifying SSA.

Cultured cells/organs, laboratory animals and embryonating eggs are widely used for isolation, propagation and identification of viruses. Frequently, viruses can be isolated and propagated on cells/tissues from their respective hosts. For example, chicken embryo fibroblasts, kidney cells, liver cells, etc., can be and have been used for isolating and propagating a number of pathogenic viruses. However, the situation appears more complex with enteric viruses which more often require a similar-target cell/tissue. The inability to grow these specialized cells is a major concern for certain cell types. This is especially true for intestinal epithelial cells. These highly specialized cells have not been grown *in vitro* using conventional techniques. This has been attributed to the multiplication/differentiation/ontogeny peculiar to these cells. A technique was developed for culturing the intestinal epithelial cells of turkeys, whereby the intestinal fibroblast cells were used as a feeder layer to nurse the epithelial cells. This technique was successful in maintaining the epithelial cells *in vitro* for periods greater than 10 days. The SSA was successfully isolated in these cultured intestinal epithelial cells. Although the SSA was successfully isolated in the primary cultures of intestinal epithelial cells, this technique has a number of limitations. The preparation of intestinal epithelial cell cultures is laborious and resource intensive. Therefore, the use of these cells on a regular basis is not feasible. Moreover, the cells do not form a confluent monolayer which makes assessing viral infectivity (by CPE) difficult. Other methods to assess viral infectivity such as the use of EM may be employed but this requires a considerable viral titer and may be cumbersome. Unfortunately, the continuous cell lines, which were used in this study, did not support SSA replication. These cell lines were selected on the basis of previous reports for their use in the isolation and propagation of enteric viruses in avian and other species. A number of methods

which enhance or allow virus propagation to occur *in vitro* were attempted. The methods were selected on the basis of successful results using other enteric agents. No adventitious agents (viruses, bacteria and/or mycoplasma) were detected in primary cells, IECs, and continuous cells following five blind passages. Although this is not a definitive proof that there were no other virus(es) present in the inoculum, it does support the fact that agent(s), which typically are expected to propagate under such conditions, were not detected. The search for continuous cell(s) for the propagation of SSA is highly desired due to their relative inexpensive maintenance, easy accessibility as well as for large scale propagation of the virus.

One of the parameters used to define SS in turkey poults and embryos is the reduction in the intestinal disaccharidases activities following infection. The intestinal disaccharidases include a number of enzymes such as maltase-glucoamylase, sucrase, isomaltase, lactase, glucosidase, trehalase, etc. (232). These enzymes are located on the microvilli of the enterocytes and show a spatial expression along the crypt-to-villus axis. For example, in mice, sucrase is not detected in the crypt of the villi but maximal activity is observed along the middle and lower portions of the villi (35, 133). However, the tip of the villi has very low levels of the enzyme. These enzymes have been detected in chicken embryos as early as 10-12 days of incubation and, depending on the individual enzyme, their activity level changes during the rest of the embryonation and following hatch (142, 239). The ontogeny of disaccharidases in turkey embryos is not known but the enzyme activity has been observed at 2 days following hatch (230). The cause of reduction in the activity of maltase, a brush border disaccharidase, in turkey poults and embryos inoculated with SS inoculum and SSA is not known. The loss of villus epithelial cells, a characteristics of some enteric viral infections, might be responsible for this reduced activity. However, the loss of epithelial cells is not extensive in SS infected poults and embryos when examined histologically (Dr. Harley Moon; Pers. comm.). The

"inapparent" loss of epithelial cells and their replacement by the less mature cells from the dividing cryptic cells (crypt depth increases following SS & SSA infection; Dr. Harley Moon; Pers. comm.) could lead to reduced intestinal maltase activity. Many studies have shown that starvation also leads to a decline in brush border proteins and sucrase-isomaltase activities (104, 150, 285). This could partially explain the reduction in maltase activity in SS affected poults as they preferentially go off feed and/or eat litter (malnutrition). But the role of malnutrition in reduced maltase activity in SSA infected embryos is difficult to explain. Reduction in intestinal maltase activity has been reported in other enteric viral infections. For example, experimental infection of piglets with transmissible gastroenteritis virus resulted in decreased sucrase and lactase activities (210). It has been reported that inflammatory mediators such as histamine, prostaglandins, etc., result in decreased sucrase and maltase activity (34, 49, 88, 191). Although the nature of the mechanism(s) of reduction in intestinal disaccharidases remain obscure, the reduced enzyme activity may lead to increased malabsorption of carbohydrates resulting in symptoms of diarrhea, gas accumulation and weight loss.

The embryonating egg has long been used for the isolation of many pathogens. The embryo provides a wide variety of cell types and/or tissues that support the growth of many different viruses. Propagation of SSA in turkey embryos was successful. The embryos were susceptible to infection when inoculated via a specific route (amniotic route). Chicken embryos were refractory to SSA infection by the same inoculation route. Moreover, the alterations produced by SSA in embryos were similar to SS infected poults. Therefore, the embryo provided an experimental model for studying the disease. It not only provided the vehicle for viral propagation but also opened venues for future research such as studies on virus neutralization, viral physico-chemical properties, etc.

It was also observed that turkey embryos inoculated with SSA had age susceptibility. This age susceptibility has also been observed in turkey poults. This could be due to the presence/absence of receptor(s), stage of cellular differentiation and maturation, physiological status of the gut, maturation of the host immune system, etc. The presence and/or absence of specific receptors explains the difference in age susceptibility for certain diseases caused by bacterial toxins and viruses. The receptors for *Escherichia coli* enterotoxin *Sta* are more abundant in the immature intestines in pigs, rats and humans (30, 31, 166). Similarly, cholera toxin exhibits enhanced binding to the intestinal microvilli of neonatal when compared with adult rabbits (20). These findings correlate with the severity of disease within various age groups. Conversely, the toxins of *Shigella* and *Clostridium difficile* have reduced microvilli binding in neonatal rabbits compared to adults and consequently, the young rabbits are less susceptible to the effects of toxin. The glycolipid receptor for *Shigella* toxin is undetectable during the suckling period but increases dramatically at the time of weaning (53, 168, 169). The rotavirus receptor has also been shown to be more abundant in the infant rat intestine than in the adult rat intestine as the disease is less severe in adults (12). The temporal expression and spatial distribution of a 200 KDa protein receptor for transmissible gastroenteritis virus (TGEV) in the intestine of pigs partially explains the pattern of age susceptibility to TGEV infection (270). The presence and/or absence of receptor(s) "partially" explains the pattern of age susceptibility in some enteric disease conditions as some other factors are likely to be involved. These include age dependent protease expression (89), age dependent mucin expression (27, 32, 291, 292, 293), age related changes in the dynamics of intestinal epithelial cell renewal and fluid absorption (170, 236), status of the host immune system (180), etc. The embryo model may provide a useful tool in resolving issues relating to age susceptibility. Studies such as sequential isolation of cells at different time and

analyzing them for presence of specific viral receptors should help to delineate the receptor concept. Immunohistochemical localization of differentiation/maturation antigens may also provide insight into the age susceptibility issues. Such studies may be of paramount importance in devising control (management & prophylaxis) strategies since there is a narrow window of time when poults are susceptible to SS. Such studies can be performed in poults but advantages of the embryo model include sterile embryo gut environment, absence of feed contents, gut flora, etc.

There are few pathophysiologic studies on many of the enteric diseases. This may be due in part because of the lack of an experimental model. The embryo provides an excellent model for studying the pathophysiology of SS and potentially other viral infections that could be adapted to embryos. It was observed that turkey embryos infected with SSA have increased intestinal fluid (diarrhea) and reduced intestinal maltase activity (chapter 5). Studying the mechanism of fluid accumulation may provide insight into developing prevention and control strategies. Infectious diarrhea is generally categorized (on the basis of mechanisms) into two classes; secretory diarrhea and malabsorbative diarrhea. Toxins (mostly bacterial) generally produce secretory diarrhea. Toxins bind to the intestinal cell receptors causing them to actively secrete. Recently, some additional factors have been suggested to be involved. These additional factors include leukocytes and their products. A combination of these two factors have been reported to induce pathology, by altering intestinal secretion and affecting malabsorption (136). This has been observed during infections with *Escherichia coli* (O157:H7) (55), *Clostridium difficile* toxins (253, 254), ameba (148), cryptosporidia (5, 6), *salmonella spp.* (23, 74), Shigella (263, 264), etc. The intestinal epithelial cells produce a variety of proinflammatory cytokines including tumor necrosis factor-α (TNF- α) (7, 107, 122, 253), interleukin-1 (IL-1) (107, 250), IL-6 (107, 238), IL-8 (52, 107), interferon-γ (INF- γ) (107), monocyte chemoattractant protein-1 (MCP-1) (107), platelet

activating factor (PAF) (62, 92), phospholipase A-II (PLA-II) (96, 145, 178, 266) etc. The invasion and/or colonization of the intestine by a pathogen results in the release of various cytokines and proinflammatory mediators which are strong chemoattractants for neutrophils, mast cells, monocytes, and lymphocytes. In turn these cells produce cytokines, lipid mediators (thromboxane, prostaglandins, leukotrienes, PAF), and histamine which results in malabsorption, secretion, villus atrophy, and crypt hyperplasia (25, 73, 138, 225, 245). It was previously hypothesized that cell loss at the proximal villi was the driving force behind cryptic hyperplasia. Currently, there is evidence to suggest that these inflammatory mediators are partly responsible for cryptic hyperplasia. The reduction in villous cell number and expansion of the cryptic cell population disturbs the equilibrium between the absorptive and secretory process. This leads to more cells having the secretory epithelial phenotype. Does a similar mechanism(s) prevail during enteric viral infection? To date, this has not been determined, however, there is evidence that support this mechanism. (see below).

The mechanism(s) of the pathogenesis of diarrhea in enteric viral infection is not yet known. However, three concepts have been postulated to explain fluid loss during viral infection of the gut. These concepts have stemmed from research on rotaviruses. They are as follows: 1). There is malabsorption secondary to enterocyte death which leads to osmotic diarrhea (32). 2). Diarrhea due to villus ischemia. Adult mice inoculated with homologous or heterologous rotavirus developed very mild lesions and diarrhea but the lesions and disease was more severe in neonates when compared to adults (29, 183, 197, 246). This suggests that diarrhea is not due to malabsorption following villus destruction. Villus ischemia was evident on histopathological examination. It has been proposed that the epithelial cells infected with rotaviruses released some vasoactive amines leading to villus ischemia which in turn resulted in functional damage without the loss of villi (183). 3). Diarrhea due

to virotoxins. Oral inoculation of mice with very high doses of psorlan-inactivated rotavirus resulted in diarrhea (235). It has recently been reported that intraperitoneal or intraileal inoculation of mice with the rotavirus nonstructural protein-4 (NSP-4) resulted in diarrhea (10). The diarrhea was of shorter duration than the actual viral infection. NSP-4 caused an increase in intracellular $[Ca^{+}]_i$ and cAMP induced Cl^{-} secretion (258, 259). This suggests that NSP-4 acted like a bacterial toxin leading to secretory diarrhea.

SSA infected embryos do experience malabsorption (decreased D-xylose absorption) however, the malabsorption is very mild (it takes longer to absorb the same level of D-xylose). Additionally, the insult to the epithelium is very mild and subtle based on the histopathological studies (data not shown). Histologically, there was no loss of villi in SSA infected embryos. Malabsorption could not be attributed to villus loss in infected poults or embryos. Is a viral toxin(s) involved in SSA infected embryos? Further research should provide insight to this question. Techniques used to study signal transduction (measurement of $[Ca^{+}]_i$ fluxes in the cells) might aid in revealing this mystery. The use of enzyme inhibitors to inhibit the enzymes involved in signal transduction pathways may also be useful. The embryo model appears to be best suited for such studies.

The immune/inflammatory mediators which induce diarrhea is another possibility to explain diarrhea during SS. The excessive fluid secretion into the intestinal lumen has been attributed to cytokines, interleukines, oxygen radicals, nitric oxides and related compounds, inflammatory mediators, etc. (also see above). The status of inflammatory mediators in the intestine during infection has not been determined. However, it was found that the intestinal intraepithelial lymphocytes (iIELs) isolated from turkey poults inoculated with SS inoculum are metabolically active (increased MTT reduction) and have increased activity of serine esterase enzyme. The insulted/damaged (i.e., virally infected) intestinal epithelial cells might

cause activation of the iIELs. Or the integrity of the intestinal barrier may be compromised due to infection resulting in exposure of iIELs directly to environmental gut antigens which cause their activation. Irrespective of how iIELs become activated, the iIELs have been reported to secrete a variety of lymphokines. Some of these lymphokines have been found to cause fluid secretion into the gut lumen. Studying the role of lymphokines in secretory diarrhea produced by viral enteritis may help in developing control strategies.

The loss of integrity of the intestinal barrier (which inhibits the passage of intact lumenal macromolecules across the gut epithelium) poses another problem. This has been observed in viral enteric infections where there was absorption of intact proteins (e.g., bovine serum albumin, horse radish peroxidase, lactalbumin) across the gut epithelium (101, 112, 121, 224). If this is the case, than these absorbed proteins might lead to hypersensitivity reactions and other immunological altercations which may have unwanted consequences. The pathophysiology of enteric infections, therefore, may be more complex than once thought.

The embryo model also provides a tool to study (in vitro) control strategies, characterization of the infectious agents, etc. During the present studies, turkey embryos were used to perform the serum virus neutralization test. Since the amniotic route was used to test the non-neutralized virus, the results can be extrapolated to design control strategies for poults. For example, by achieving the effective concentration of virus specific antibodies in the intestinal lumen, the virus infectivity may be neutralized (passive protection). The effective concentrations of antibodies needed may be provided either orally (e.g., feed), parenterally (e.g. by subcutaneous injection), by maternal antibodies (via the hen) and/or combination of these.

The alterations in turkey embryos infected with SSA are similar to poults infected with SS. The intestinal maltase activity and D-xylose absorption were

reduced in infected embryos. The intestinal lesions included pale, thin and fragile intestines filled with fluid. These were very similar to what was seen in SS affected turkeys. The embryo model was not only an economical alternate but also offered an advantage of having a short (72 h) experimental time. Additionally, the embryo model does not meet the criteria for the use of live animals as defined by the current regulations. Therefore some ethical issues pertaining to animal usage may be avoided.

The SSA is a pleomorphic, membraned virus ranging in size from 60 to 95 nm in diameter. The viral nucleocapsid appears either round, kidney-bean-shaped, dumbbell-shaped or bacilli-shaped. A similar kind of morphology has been reported for toroviruses, a distinct genus of viruses contained within the family *Coronaviridae*. The members of the torovirus genus possess kidney-bean-shaped, bacilli-shaped, dumbbell-shaped, or circular morphology. Although, the SSA appears morphologically similar to toroviruses, it was serologically distinct from bovine breda-1 and bovine breda-2 viruses. Bovine breda viruses are members of the genus torovirus and are antigenically related to other members of the torovirus genus (berne virus, human torovirus, etc.). At this point it would be premature to conclude that the nature of SSA is a torovirus solely on the basis of morphological resemblance. However, the avian infectious bronchitis virus (a member of the coronavirus genus) has been placed in a separate antigenic group despite its morphological resemblance to other coronaviruses in the coronavirus genus. The antigenic uniqueness of SSA from other avian viruses strongly suggests that SSA is a distinct pathogen.

The family *Coronaviridae* has been divided into two genera; coronavirus and torovirus genus. The avian members of the genus coronavirus are infectious bronchitis virus (123) and turkey coronavirus (bluecomb disease) (194). To date no avian member of genus torovirus has been described. The coronaviruses are

pleomorphic, enveloped viruses with roughly spherical shape (helical nucleocapsid). The toroviruses are disc-, kidney-, or rod shaped (tubular nucleocapsid) (125). There is little sequence similarity between coronavirus and torovirus proteins. The N protein of coronavirus is larger than that of torovirus (125). The hemagglutination profile of coronaviruses varies with different member viruses. SSA hemagglutinated erythrocytes from rat blood. Bovine Breda viruses (torovirus) also hemagglutinate rat erythrocytes while the turkey coronavirus does not (125). The physico-chemical properties of SSA is typical of an enteric virus. Therefore, further research is needed for proper classification of SSA.

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